MOLECULAR CHARACTERIZATION OF THE GENE, mRNAS, PRECURSOR PROTEINS, AND MATURE SUBUNITS INVOLVED IN THE SYNTHESIS OF THE NADP-SPECIFIC GLUTAMATE DEHYDROGENASE ISOENZYMES IN *CHLORELLA SOROKINIANA*

By

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This work is dedicated to my parents, Walter and Elaine Miller, who taught me by example that success is only achieved by dedication and hard-work, and that true success can be measured in many ways.

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LIST OF ABBREVIATIONS

AP	Alkaline phosphatase
ATCase	Aspartate trans-carbamylase
BSA	Bovine serum albumin
ca	Calculated average
CaM	Calmodulin
CPSase	Carbomylphosphate synthase
DHOase	Dihydroorotase
DTT	Dithiothreitol
Dsx	Double-sex
EDTA	Ethylene diamine-tetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N, N, N', N',-tetraacetic acid
ELISA	Enzyme-linked immuno-absorption assay
GS	Glutamine synthetase
GOGAT	Glutamate synthase
HAT	Hypoxanthine-aminopterin-thymidine
HCR	Highly conserved region
ICBR	Interdisciplinary Center for Biotechnology Research

LANT6	Neurotensin lysine-asparagine rich
mA	Milliamperes
MAb	Monoclonal antibody
NAD-GDH	Nicotinamide adenine dinucleotide-specific glutamate dehydrogenase
NADP-GDH	Nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase
NBT	Nitroblue tetrazolium
NEB	New England Biolabs
NMN	Neuromedian N
nt	Nucleotides
NT	Nerotensin
ODU	Optical density unit(s)
OTCase	Ornithine trans-carbamylase
PK	Pyruvate kinase
PME	Pectin methylesterase
RACE-PCR	Rapid amplification of cDNA ends-PCR
RPA	Ribonuclease protection assay
RT-PCR	Reverse transcriptase PCR
Rubisco	Ribulose bisphosphate carboxylase/oxygenase
SNAP-25	Synaptosomal associated protein 25 kD
snRNA	Small nuclear ribonucleic acid
snRNP	Small nuclear ribonuclear proteins

SPP	Stromal processing peptidase
SPT	Serine:pyruvate amino-transferase
Sxl	Sex-lethal
TBS	Tris-buffered saline
tra	Transformer
TTBS	Tween Tris-buffered saline
UTR	Untranslated region
VR	Variable region
X-phosphate	5-bromo-4-chloro-3-indole phosphate

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

MOLECULAR CHARACTERIZATION OF THE GENE, mRNAS, PRECURSOR PROTEINS, AND MATURE SUBUNITS INVOLVED IN THE SYNTHESIS OF THE NADP-SPECIFIC GLUTAMATE DEHYDROGENASE ISOENZYMES IN *CHLORELLA SOROKINIANA*

By

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Chairman: Dr. Robert R. Schmidt
Major Department: Microbiology and Cell Science

Chlorella sorokiniana possesses seven chloroplastic NADP-glutamate dehydrogenases (NADP-GDHs) composed of varying ratios of α- and β-subunits. Southern blot analysis and allele-specific PCR demonstrated the *C. sorokiniana* genome possesses a single NADP-GDH gene encoding both the α- and β-subunits. PCR analysis, cDNA cloning and sequencing, and RNase protection analysis identified two NADP-GDH mRNAs that are identical with the exception of a 42 nt insert located in the 5' coding region of the longer mRNA. Deduced amino acid sequence analysis revealed that the 42 nt insert encodes an additional 14 amino acids. The absence or presence of the insert does not affect the downstream reading frame. The +42 nt mRNA encodes a 53850 D precursor protein, whereas the -42

nt mRNA encodes a 52350 D precursor protein. The +42 nt and -42 nt mRNAs are postulated to be derived via alternative splicing of a pre-mRNA from the single 7.1 kbp NADP-GDH gene that consists of 22 or 23 exons, respectively.

Western blot analysis of the α- and β-subunits showed them to be antigenically similar and to be 53.5 and 52.3 kD in size, respectively. Amino-terminal sequence analysis revealed the α-subunit shares amino acid sequence identity with the β-subunit; however, the α-subunit possesses a unique 11 amino acid α-helical domain that is lacking in the β-subunit.

The induction patterns and relative abundances of the NADP-GDH mRNAs, antigens, and activities were measured in cells induced in 29 mM ammonium medium. The relative abundance of the +42 nt mRNA correlated with the α-subunit antigen, whereas the -42 nt mRNA correlated with the β-subunit. The ratio of NADPH:$NADP^+$-GDH activity was highest when the β-subunit was prominent and lowest when the α-subunit was prominent.

These results are consistent with a single nuclear gene being transcribed into a pre-mRNA that is alternatively processed to yield two mRNAs encoding two precursor proteins. The precursor proteins are processed to either the α- or β-subunit and assembled into isoenzymes with varying ammonium-affinities.

INTRODUCTION

Inorganic nitrogen acquired by plants is ultimately converted to ammonium before being assimilated in organic nitrogen metabolism. One of the enzymes postulated to be involved in the assimilatory process is GDH, a ubiquitous enzyme found to be present in almost all organisms from microbes to higher plants and animals (Srivastava and Singh, 1987). GDH catalyses the reversible conversion of α-ketogluterate to glutamate via a reductive amination that utilizes NADH or NADPH as a cofactor. The role of plant GDHs in the assimilation of ammonium into amino acids has been questioned since the discovery of the GS/GOGAT pathway that is believed to be the favored pathway for ammonium assimilation in higher plants (Miflin and Lea, 1976). The primary objection to GDH playing a major role in nitrogen metabolism is its low affinity for ammonium that would require high intracellular ammonium concentrations to function anabolically. Early evidence indicated that GDH is a catabolic enzyme catalyzing the deamination of glutamate with only a partialy anabolic function in synthesizing glutamate (Wallsgrove et al., 1987). However, more recent studies reveal that K_m values for ammonium and other sustrates may be affected by various internal and external factors and the previously reported *in vitro* K_m values may

not reflect *in vivo* conditions. The physiological role of large amounts of GDH present in various plant tissues and organelles is still unclear, and possible conditions under which GDH may play a significant role in carbon and nitrogen metabolism have not been resolved.

The majority of plant GDHs characterized to date are localized in the mitochondria; however, GDH species differing in several properties (i.e. cofactor specificity) have been characterized from chloroplasts (Srivastava and Singh, 1987). *Chlorella sorokiniana* cells have been shown to possess a constitutive, mitochondrial, tetrameric NAD-specific GDH (Meredith et al., 1978), and seven ammonium-inducible, chloroplast-localized, homo- and heterohexameric NADP-specific GDH isoenzymes (Prunkard et al., 1986; Bascomb and Schmidt, 1987). The seven chloroplastic NADP-GDH isoenzymes were shown to have different electrophoretic mobilities during native-PAGE, and presumably result from the formation of homo- and heterohexamers composed of varying ratios of α- and β-subunits (53.5 and 52.3 kD, respectively). *Chlorella* cells cultured in 1 to 2 mM ammonium medium accumulate only the α-homohexamer (Bascomb and Schmidt, 1987). The addition of higher ammonium concentrations (3.4 to 29 mM) to nitrate-cultured cells results in the accumulation of both α- and β-subunits in NADP-GDH holoenzymes (Prunkard et al., 1986; Bascomb and Schmidt, 1987; Bascomb et al., 1987). Prunkard et al. (1986) demonstrated that the NADP-GDH subunit ratio and isoenzyme pattern is influenced by both the carbon and

nitrogen source as well as the light conditions under which cells are cultured.

The purified α- and β-homohexamers have strikingly different ammonium K_m values; however, the K_m values for their other substrates are very similar. The α-homohexamer is allosterically regulated by NADPH and possesses an unusually low K_m for ammonium that ranges from 0.02 to 3.5 mM, depending on the NADPH concentration (Bascomb and Schmidt, 1987). In contrast, the β-homohexamer is a non-allosteric enzyme with an ammonium K_m of approximately 75 mM. It is postulated that the heterohexamers have varying degrees of affinity for ammonium; however, no kinetic analyses have been performed on purified heterohexamers. Pulse-chase experiments, performed when homo- and heterohexamers of NADP-GDH were accumulating during early induction in 29 mM ammonium medium, revealed the α-subunit antigen was degraded with a half-life of 50 min whereas the β-subunit antigen was degraded more slowly with a half-life of 150 min (Bascomb et al., 1986). After the removal of ammonium from the induced cells, enhanced rates of degradation were observed for the α- and β-subunit antigens, half-lifes of 5 and 13.5 min, respectively.

Although the α- and β-subunits have distinct *in vivo* turnover rates and the corresponding homohexamers have remarkably different ammonium K_m values, the α- and β-subunits are derived from precursor proteins of nearly identical size (ca 58,000 D) and were shown to have very

similiar peptide maps (Prunkard et al., 1986; Bascomb and Schmidt, 1987). Moreover, antibodies prepared against the β-homohexamer are capable of immunoprecipitating all of the NADP-GDH isoenzymes (Yeung et al., 1981, Bascomb et al., 1987), but do not crossreact with the mitochondrial NAD-GDH. In addition, previous research in this laboratory provided genomic cloning and southern blot evidence that indicated the *C. sorokiniana* genome possesses a single NADP-GDH structural gene (Cock et al., 1991).

Biochemical and immunochemical properties of the NADP-GDH α- and β- subunits suggest that the two subunits share a significant amount of protein sequence identity. Similar kinetic, isoenzyme pattern, and immunological properties have been shown for the mitochondrial GDH of grapevine (Loulakakis and Roubelakis-Angelakis, 1991) and Arabidopsis (Cammaerts and Jacobs, 1985). The understanding of the molecular mechanisms regulating the *C. sorokiniana* NADP-GDH isoenzymes is critical to further elucidate the metabolic significance of GDH in carbon and nitrogen metabolism in *Chlorella* and higher plants. Therefore, the purpose of this study is to determine if the two NADP-GDH subunits arise from the (i) differential processing of a precursor protein encoded by a single nuclear gene and mRNA, (ii) specific processing of two similar precursor proteins encoded by two mRNAs formed by alternative splicing of a pre-mRNA derived from a single nuclear gene, (iii) specific processing of two precursor

proteins encoded by two mRNAs transcribed from two closely related nuclear genes.

LITERATURE REVIEW

Extensive research into the molecular and biochemical mechanisms that control the physiology and potential fate of a living cell has revealed a myriad of complexities in the regulation of cellular processes. In prokaryotes, metabolic processes have been shown to be temporally regulated at transcription, translation, mRNA turnover and processing, and post-translational modifications. The presence of organelles in eukaryotes has provided another level of intricacy in metabolic regulation by providing compartmentalization, that provides for both temporal and spacial separation of cellular events. The spatial separation of cellular processes provides additional steps where regulation of transriptional, post-transcriptional, translational, and post-translational events can occur.

In response to the spatial and temporal separation of metabolism, cells have evolved enzymes that share a similar biological activity within an organism. These enzymes often differ in their primary amino acid sequence (isoenzymes or isoproteins), but may be capable of subunit exchange (isozymes). Enzymatically similar isoforms may also exist as a result of post-translational modifications (phosphorylation, acetylation, methylation, etc.) to a single protein. Theoretically, multiple isoenzymes have allowed

organisms to respond differentially in a refined way to a broader range of developmental and environmental conditions. Isoenzymes can arise via all the aforementioned cellular processes and provide a useful tool to study the molecular mechanisms involved in regulating biochemical processes.

The majority of isoenzymes characterized to date are encoded by two or more genes within the genome of a organism. Isoenzymes encoded by multiple genes are believed to have arisen through gene duplication within an organism or via gene exchange between organelles of eukaryotes (Gray and Doolittle, 1982; Sun and Callis, 1993). The amount of similarity conserved among isoenzymes derived from different genes is influenced by how recent the duplication or exchange has occurred (Pickersky et al., 1984), or may reflect a strong selective pressure to maintain the primary amino acid sequence (Moncreif et al., 1990).

Multiple isoenzymes of CaM, a 16 kD acidic Ca^{2+}-binding, signal transducing protein, have been identified in all eukaryotes examined (Ling et al., 1991). Increased binding of Ca^{2+} by CaM in response to increased intracellular Ca^{2+} levels triggers a conformational change in the protein. Alteration of conformation in turn facilitates specific interactions with Ca^{2+}/CaM-dependent enzymes (O'Neil and DeGrado, 1990). One notable feature of CaM isoenzymes is the highly conserved primary structure; comparisons of amphibian, avian, mammalian, and plant CaM isoproteins showed identities of over 90 percent (Roberts et al., 1986). Analysis of CaM

amino acid sequences from a wide variety of organisms has revealed that 47 of 148 amino acid residues are variant, allowing a degree of latitude in the physiological constraints that regulate the structure and function of each CaM isoenzyme (Moncreif et al., 1990).

CaM multigene families have been characterized in rat (Nojima, 1989), and human (Fischer et al., 1988). In both of these species the CaM proteins encoded by different gene families possess identical amino acid sequences; however, their respective nucleotide sequences have diverged by approximately 20 percent (Ling et al., 1991). More recently, at least four CaM isoforms have been identified in *Arabidopsis thaliana* that differ from one another by as much as six amino acid substitutions (Gawienowski et al., 1993).

Arabiodopsis CaM isoenzymes are encoded by a multigene family consisting of at least six different genes. Southern blot analysis and genomic cloning determined the CaM proteins were not allelic and that their coding regions had diverged thirteen to twenty percent, and no significant identities were retained in their mRNA 3'-untranslated regions. Most of the amino acid changes between the CaM proteins appear to be functionally conservative, and are clustered within the fourth Ca^{2+}-binding domain that is involved in high affinity Ca^{2+} binding. It has yet to be determined if there exists significant biochemical differences or tissue-specific expression differences that would warrant these multiple CaM proteins (Gawienowski et al., 1993). Multiple CaM isoforms,

each having a defined set of targets, may explain how a common intracellular Ca^{2+} concentration signal can activate different physiological responses.

In eukaryotes, multiple isoenzymes of CPSase and ATCase, enzymes involved in the *de novo* pyrimidine biosynthetic pathway have been identified (Jones, 1980; Ross, 1981). Isoenzymes of these proteins have proven to be critical in the biochemical regulation of eukaryotic pyrimidine and arginine biosynthesis, both of which utilize carbomylphosphate as an intermediate. Prokaryotes possess a single CPSase and ATCase enzyme, both of which are metabolically regulated by negative effectors to control flux of carbomylphosphate between the two competing pathways (Markoff and Radford, 1978).

Eukaryotes, other than plants, utilize two isoenzymes of CPSase to commit separate pools of carbomylphosphate to the pyrimidine and arginine pathways. An arginine-specific CPSase is localized in the mitochondria with OTCase, the enzyme that utlizes carbomylphosphate to synthesize citrulline in the arginine pathway (Davis, 1986). A pyrimidine-regulated CPSase activity exists on a multifunctional protein that also exhibits ATCase activity and is localized in the nucleus of yeast (Nagy et al., 1989), or a cytosloic localized multifunctional protein that also exhibits ATCase and DHOase activity observed in other eukaryotes (Davidson et al., 1990). The different CPSase

activities have been shown to be encoded by separate genes in these organisms.

The mechanisms of coordinately regulating the pyrimidine and arginine pathways of plants are less understood. Sequence analysis of partial cDNA clones from alfalfa has provided evidence for the existence of arginine- and pyrimidine-specific CPSases (Maley et al., 1992); however, biochemical studies have only demonstrated a single glutamine-dependent CPSase activity. Regardless of the number of CPSases in plants, metabolic studies indicate that the arginine and pyrimidine pathways share a common pool of carbomylphosphate (Lovatt and Cheng, 1984). Localization of CPSase, ATCase, and OTCase activities to the plant chloroplast indicates that allocation of carbomylphosphate to each pathway must be regulated (Shibata et al., 1986).

Williamson and Slocum (1994) utilized an ATCase deficient mutant of *Escherichia coli* to clone by functional complementation two different ATCases from pea plants. Comparison of the deduced amino acid sequences of the clones revealed an 85 percent identity and indicated they both possessed a chloroplast targeting transit peptide. Southern blot analysis revealed the two ATCase mRNAs are encoded by two independent genes of the pea genome. Biochemical studies are in progress to determine if these genes are differentially regulated and if different ATCase subunits which exist as homotrimers can also exist as heterotrimers with unique kinetic properties.

Cell wall PME enzymes that de-esterify galactosyluronic acid units of pectin, have been detected in all tissues of higher plants analyzed. PME has been implicated in functioning in a broad range of cellular processes including fruit softening (Fischer and Bennett, 1991), plant response to infection (Collomer and Keen, 1986), and cell growth (Moustacos et al., 1991). Multiple isoenzymes of PME have been detected in most plant species and tissues. It is hypothesized that the multiple isoenzymes function in a tissue-specific manner and have different modes of de-esterification of pectins (Markovic and Kohn, 1984).

Harriman et al. (1991) and Recourt et al. (1992) demonstrated that tomato and *Phaseolus vulgaris* genomes possess multiple PME genes. The cDNAS isolated from tissue-specific libraries indicated that there are multiple PMEs with high sequence homologies encoded by separate genes. *In vivo* expression of antisense RNA constructs, designed to block specified PME isoforms, revealed regulation of PME isoenzymes occurs by regulating transcription from different genes in a developmental, tissue-specific manner (Gaffe et al., 1994).

Multiple isoproteins do not exist in all cases where multiple genes are detected. Multiple genes encoding (1-3,1-4)-β-glucanendohydrolases have been identified in wheat (*Triticum aestivium*). Isolation of β-glucanase cDNA clones revealed two different cDNAs with 31 nucleotide substitutions; however, the coding regions of the mRNAs were

identical and only a single β-glucanase protein was detected. Further analysis revealed that the two mRNAs originated from homeologous chromosomes in the wheat hexaploid genome (Lai et al., 1993). Therefore, the potential exists to derive multiple isoenzymes by combining genomes in polyploid species. The level of conservation of similar proteins will be determined by relatedness of the parental species, the time lapse since the genomes were combined, and selective pressures to maintain the primary structures of the proteins.

Multiple isoenzymes have been shown to be derived from a single gene in an organism. *Saccharomyces cerevisiae* cells possess both a cytosolic and secreted form of invertase. Both isoenzymes of invertase are encoded by a single structural gene, SUC2, which gives rise to two distinct mRNA species (Perlman and Halvorson, 1981; Carlson and Botstein, 1982). The polypeptides encoded by the invertase mRNAs, when translated *in vitro*, are 60 kD (p60) and 62 kD (p62). The p60 mRNA is 1.8 kb and encodes the cytoplasmic invertase, whereas the p62 mRNA is 1.9 kb and encodes the secreted invertase (Carlson and Botstein, 1982). The p62 form has been shown to be glycosylated in the Golgi to an 87 kD protein, a process that targets the invertase for secretion. The p62 protein is preferentially targeted to the Golgi apparatus via a labile 19 amino acid amino-terminal signal sequence that is cotranslationally cleaved upon import to the Golgi (Perlman et al., 1982).

Amino acid analysis of the p60 and p62 proteins revealed that, starting at amino acid residue 21, the secreted invertase was identical to the cytoplasmic invertase. Comparison of the 5' ends of the mRNA nucleotide sequences and the SUC2 gene revealed the presence of two unique promoters (Tassig and Carlson, 1983; Sarokin and Carlson, 1984, 1985). The promoter region for the secreted invertase was located -140 bp upstream of the coding region, whereas the intracellular invertase promoter was located -40 bp upstream of its coding region. Deletion of the nucleotide sequence from -650 to -418 bp removed the regulation of the secreted form by glucose; however, deletion from -1900 to -80 bp had no influence on the cytoplasmic invertase (Sarokin and Carlson, 1984).

Mammalian SPT is localized in two subcellular organelles, the mitochondria and peroxisomes of the liver. The peroxisomal SPTp and the mitochondrial SPTm isoenzymes have very similar immunochemical (Oda et al., 1982), catalytic and physical properties (Naguchi and Takada, 1978), but their responses to hormones or other stimuli are quite different. The SPTm is synthesized as a large precursor which is specifically translocated into the mitochondria, both *in vivo* and *in vitro*, and is processed into a mature form similar in size to the mature SPTp. Two different SPT mRNAs were detected by northern analysis using a SPTm probe. The longer 1.9 kb mRNA was glucagon inducible and the smaller 1.7 kb mRNA was hormone insensitive indicating the larger

mRNA codes for the SPTm protein (45 kD) and the smaller mRNA encodes the SPTp protein (43 kD) (Oda et al., 1993).

Cloning and sequence analysis of the two transcripts provided evidence that the two mRNAs were identical except for a longer 5' end possessed by the 1.9 kb SPTm mRNA. These results were verified by S1 nuclease protection, and RNase protection analysis. Utilizing SPTm and SPTp probes, Southern blot analysis detected a single SPT gene. Comparison of the cDNA and gene sequences revealed that the two SPT mRNAs were generated by transcription from two unique promoters located upstream of exon one. The SPTm mRNA contains an amino terminal extension of 22 amino acids that acts as a mitochondrial targeting signal, whereas the SPT mRNA lacks the targeting signal due to initiation of transcription from a different promoter downstream of the mitochondrial start methionine codon in exon one. Transport of SPTp to the peroxisome occurs if the SPT preprotein lacks the mitochondrial targeting peptide (Oda et al., 1993). There are many other examples of multliple isoproteins generated from a single gene via the use of alternate upstream promoters (Beltzer et al., 1988; Chatton et al.,1988).

Alternative splicing of pre-mRNA transcribed from a common promoter of single gene has emerged as a widespread mechanism for regulating gene expression. In most cases, alternative splicing gives rise to multiple protein isoforms that share high identity, but vary in specific domains that

allow fine regulation of protein function (Smith et al., 1989). Alternative splicing allows for protein isoform switching without the need for permanent genetic change that would be necessary with gene rearrangement. The number of genes known to be alternativly spliced reported to date is so vast; therefore, a limited number of representative cases will be discussed.

Alternative splicing of transcripts has been shown to regulate the localization of isoproteins. The immunoglogulin heavy chain protein Igμ is present as a membrane bound form in early B lymphocytes. Upon maturation of the B-cell, after antigen activiation, the membrane-bound Igμ form decreases and a concomitant increase in the Igμ secreted pentamer form is observed. The switch from membrane bound to secreted form is acheived by the alternate use of 3' end exons that encode the hydrophobic membrane-binding segment (Alt et al., 1980; Rogers et al.,1980).

Gelosin, a protein which severs actin filaments, exists as a plasma and cytosolic protein. Analysis of the two isoenzymes indicated the proteins were identical except for an additional 25 amino-terminal amino acids in the plasma form. Analysis of the gelosin gene revealed the two isoenzymes are expressed from the same gene via the use of alternate promoters and subsequent alternative splicing of a 5' exon. The extra 25 amino acids of the mature plasma form and an additional 27 amino acid signal peptide is encoded in the extra exon. The 27 amino acid sequence targets the

plasma form to a secretion pathway and is cleaved from the mature protein (Kwiatkowski et al., 1986).

Alternative pre-mRNA splicing can also function to produce a functional and nonfunctional form of a protein that acts as an on/off switch. The pathway of sexual diffentiation in *Drosophilia* has revealed that an entire sexual developmental cascade is regulated by alternative splicing (Bingham et al., 1988). Briefly reviewed here, in response to the X chromosome:autosome ratio this pathway is regulated initially by the alternative splicing of three primary genes:*Sxl*, *tra*, and *dsx*.

The *Sxl* gene, the first gene in the cascade, pre-mRNA is alternatively spliced to yield male and female specific transcripts. The splicing results in the inclusion of an exon in male transcripts and in the truncation of the major open reading frame after 48 codons. In females, the male specific exon is spliced out and a complete 354 amino acid RNA-binding protein is produced (Bell et al., 1988). The functional female *Sxl* protein regulates the splicing out of a 248 bp intron in the *tra* gene mRNA which produces a female function tra protein. The lack of the functional sxl protein product in males leads to a default splicing pattern in the male *tra* mRNA which produces a nonfunctional tra protein (Boggs et al., 1987; Nagoshi et al., 1988). The functional tra protein of females regulates the splicing of the *dsx* pre-mRNA to a female-specific transcript that encodes a dsx protein that represses male differentiation genes.

Alternative splicing of the *dsx* pre-mRNA produces functional products in males and females; however, the male *dsx* mRNA possesses a male specific exon that is spliced out of the female mRNA. The male dsx protein represses the female diferrentiation genes (Baker and Wolfner, 1988). Further analysis revealed that the functional tra protein of females acts by recruiting general splicing factors to a regulatory element downstream of the female-specific 3' splice site of the *dsx* mRNA (Tain and Maniatis, 1993).

Changes in the enzymatic activity of the enzyme PK can be attributed to mRNA alternative splicing. The four isoforms of PK, M1 and M2, L and R, each form homotetrameric holoenzymes. The M1 and M2 isoenzymes differ by the presence of an internal 45 amino acid segment that is encoded by a pair of mutually exclusive exons (Noguchi et al., 1986). This variable region is nearly identical to a similar region found in the L and R isoforms. The M2, L, and R homotetramers are all allosterically regulated and show sigmoidal kinetics, whereas the M1 isoenzyme shows no allosterism and has Michaelis-Menton kinetics (Imamura and Tanaka, 1982). The M2, L, and R proteins reside in tissues where allosteric regulation is critical to prevent futile cycling, whereas the M1 form exists in muscle tissue in which glycolysis is the dominant metabolic state. The alternatively spliced exons of M1 and M2 encode a region that is important in intrasubunit contacts; therefore, it is postulated that interactions between subunits dictate the

regulatory properties of the individual isoenzymes (Noguchi et al., 1986).

Alternative splicing also influences post-translational modifications to isoproteins. The 25 kD synaptosomal associated protein, SNAP-25, exists as two isoforms in chicken. Cloning and characterization of the SNAP-25 cDNAs and gene revealed that two exon fives exist in the gene. Alternative splicing of the pre-mRNA mutually excluded one of the two exons that resulted in two isoproteins that differed in nine amino acid substitutions. The amino acid substitutions result in the loss of a palmitoylation site, thus influencing the ability of the isoforms to interact with neuronal membranes (Bark, 1993).

The intracellular signaling by Ca^{2+} has been shown to be finely regulated by alternative splicing in humans. The Ca^{2+} pump is a calmodulin-regulated P-type ATPase that is critical in controlling intracellular Ca^{2+} concentrations. Analysis of the pump gene structure indicated that alternative splicing of the Ca^{2+} pump pre-mRNAs altered the calmodulin-binding domain. Alteration of this domain either increases or decreases pump affinity for calmodulin. The decreased affinity for calmodulin causes an apparent lower affinity of the pump for Ca^{2+}, thus lowering the intracellular Ca^{2+} concentration (Enyedi et al., 1994).

Although alternative splicing appears to be a common mechanism of altering gene function without permanently changing gene structure, few examples of alternative mRNA

have been reported for plants. This absence of published reports likely reflects a lack of detection of alternative mRNA splicing rather than a lack of its existence. Rubisco initiates the pathway of photosynthetic carbon reduction in plants. This enzyme exhibits catalytic activity only after activation by rubisco activase. Immunoblots utilizing anti-activase antibodies detected two polypeptides in spinach (41 and 45 kD) and *Arabidopsis* (44 and 47 kD) indicating that the two polypeptides were similar (Werneke, 1988). Genomic DNA blots indicated that rubisco activase was encoded by a single gene in spinach and *Arabidopsis*. Werneke et al. (1989) demonstrated by amino- and carboxyl-terminal amino acid analysis and cDNA cloning that the two activase isoenzymes were derived by alternative splicing of activase pre-mRNA. In spinach, two different 5' splice sites are utilized in processing an intron in the 3' end of the primary transcript. Use of the first 5' splice site introduced a termination codon that results in formation of the 41 kD protein, whereas selection of the downstream 5' splice junction omits the temination codon and yields the 45 kD isoprotein. In *Arabidopsis*, alternative splicing by a similar mechanism results in the synthesis of the 44 kD and 47 kD polypeptides. However, retention of the intron sequence does not introduce a termination codon, but creates a frameshift that leads to early termination of the protein. These results represent the first case of alternative splicing reported for plants.

The P gene of *Zea mays* is postulated to transcriptionally regulate flavonoid-derived pigment biosynthesis in floral tissues. Two different P transcripts have been detected, a 1.8 kb mRNA encoding a 43.7 kD protein and a 0.945 kb mRNA encoding a 17.3 kD protein. Characterization of the two P transcripts revealed they are derived from a single gene and arise via alternative splicing of the 3' end of a pre-mRNA (Grotewold et al., 1991). The altenative splicing between exons two and three of the pre-mRNA results in a frameshift that leads to early termination of translation and yields the 17.3 kD polypeptide. The truncated protein still possesses its DNA-binding domain; therefore, it is hypothesized that the 17.3 kD protein may act as a negative regulator and inhibits binding of the 43.7 kD functional transcriptional activator (Grotewold et al., 1991).

Three cDNAs encoding RNA-binding proteins have been isolated from *Nicotiana* (*Nicotiana sylvestris*) that encode proteins with high affinities for polyuracil and polyguanine motifs. Two of the cDNAs appear to be derived from a common gene whose pre-mRNA undergoes alternative splicing in a tissue specific manner. The alternative splicing occurs via differential selection of two 5' splice junctions and results in the formation of a functional and a truncated polypeptide. The physiological significance of the isoproteins is unknown; however, the functional polypeptide shows high homology with

the RNA-binding protein involved in the dsx alternative splicing machinery of *Drosophilia* (Hirose et al., 1994).

Multiple isoenzymes can be derived by differential proteolytic processing of a common precursor protein; however, to date this mechanism has rarely been demonstrated. NT and NMN, putative endocrine and neural signal prohormones, are present in a 1:1 ratio within a common precursor preprohormone polypeptide in mammals (Dobner et al., 1987). Post-translational precursor processing has been shown to occur in a tissue specific manner to yield both NT and NMN prohormones in canines (Carraway and Mitra, 1990). Chicken NT and LANT6 are derived via differential processing of a common precursor prohormone (Carraway et al., 1993). Since both NT and LANT6 have similar pharmacological activities and bind similar receptors, it is postulated that the larger slower degraded LANT6 may produce similar effects with a different temporal pattern.

There are other translational and post-translational mechanisms that could potentially yield multiple isoforms of a protein. Such mechanisms include internal initiation of translation (McBratney et al., 1993), protein splicing (Neff, 1993), protein methylation (Clark, 1993), protein acylation, phosphorylation, and glycosylation (Blenis and Resh, 1993). Although these mechanisms of regulation are undergoing extensive research, the role and significance they play in isoprotein formation is not well documented.

GDH is a ubiquitous enzyme detected in almost all organisms from microbes to higher plants and animals (Srivastava and Singh, 1987). This enzyme catalyzes the reversible conversion of α-ketoglutarate to glutamate via a reductive amination that utilizes NADH/NADPH as a cofactor. A multitude of studies utilizing various techniques has revealed that isoenzymes of GDH can be localized within the mitochondria, chloroplast, and the cytoplasm depending on the organism (Srivastava and Singh, 1987; LeJohn et al., 1994). Multiple roles have been attributed to GDH including ammonia assimilation (Yamaya and Oaks, 1987), maintaining the glutamate/α-ketoglutarate ratio to regulate flux between carbon and nitrogen metabolism (Munoz-Blanco and Cardenos, 1989), stress response (Miranda-Ham and Loyola-Vargus, 1988; LeJohn et al., 1994), and function as a RNA-binding protein (Preiss et al., 1993). Collectively, these findings implicate GDH as playing a variety of cellular roles and these various metabolic funtions are regulated by the differential use of GDH isoenzymes.

Chlorella sorokiniana has been shown to synthesize multiple GDH isoenzymes: a constitutive, tetrameric, mitochondrial NAD-GDH (subunit 45 kD), and seven ammonium-inducible, hexameric, chloroplastic NADP-GDHs (subunit 53kD,β- and 55.5 kD,α-) (Prunkard et al., 1986; Bascomb and Schmidt, 1987). The multiple chloroplastic isoenzymes have different molecular weights and charges and presumably result from the formation of homohexamers and heterohexamers due to

mixing of the α-subunits and β-subunits. The chloroplastic isoenzyme pattern in *C. sorkiniana* has been shown to be influenced by light conditions, carbon and nitrogen source, and ammonium concentration (Isreal et al., 1977; Prunkard et al., 1986; Bascomb and Schmidt, 1987).

Kinetic and physical characterization of the purified α-homohexamer and β-homohexamer show them to have several properties in common as well as striking differences (Bascomb and Schmidt, 1987). The purified homohexamers have remarkably different affinities for ammonia; however, affinity values for the other substrates are quite similar. The α-homohexamer is an allosteric enzyme with a low K_m for ammonia depending on the NADPH concentration, whereas the β-homohexamer is nonallosteric and has a high K_m for ammonia. In addition, pulse-chase experiments demonstrated that the two subunit types are synthesized and degraded at different rates (Bascomb and Schmidt, 1987). Although the two subunit types have distinct *in vivo* turnover rates, they appear to be derived from precursor proteins of near identical size. Antibodies derived against one subunit type are able to immunprecipitate both α-subunits and β-subunits (Yeung et al., 1981), and peptide mapping of the subunits revealed that the subunits have 36 of 40 peptides in common (Bascomb and Schmidt, 1987). These results indicate that the two subunits are very similar. Genomic cloning and Southern blot analysis indicated a single NADP-GDH gene exists in the *C. sorokiniana*

genome that encodes both types of subunits (Cock et al., 1991).

In summary, multiple isoenzymes have evolved as a mechanism to allow an organism to respond to a broad range of developmental and environmental conditions in a tissue-specific manner. Isoenzymes and isoproteins can arise by a variety of molecular and biochemical events including gene duplication, alternative RNA splicing, transcriptional, translational, and post-translational events. GDH, a ubiquitous enzyme, exists as multiple isoenzymes with various roles in most organisms studied. Considering the wealth of information that exists on the metabolic roles and regulation of GDH isoenzymes, further research into the biochemical genetics of these isoenzymes may provide insight into mechacanisms of molecular regulation in general.

MATERIALS AND METHODS

Culture Conditions

C. sorokiniana cells were cultured autotrophically as previously described by Prunkard et al. (1986) in a modified basal salts medium. The modified medium contained in mM concentration: $CaCl_2$, 0.34; K_2SO_4, 6.0; KH_2PO_4, 18.4; $MgCl_2$, 1.5; in μM concentration $CoCl_2$, 0.189; $CuCl_2$, 0.352; EDTA, 72; $FeCl_3$, 71.6; H_3BO_3, 38.8; $MnCl_2$, 10.1; NH_4VO_4, 0.20; $(NH_4)_6MO_7O_{24}$, 4.19; $NiCl_2$, 0.19; $SnCl_2$, 0.19; $ZnCl_2$, 0.734. The medium was supplemented with 1 mM NH_4Cl, 29 mM NH_4Cl, or 29 mM KNO_3 as a nitrogen source depending on the experimental conditions. The medium containing NH_4Cl was adjusted to pH 7.4, and medium containing KNO_3 was adjusted to pH 6.8 with KOH after autoclaving. Cells were supplied with a 2%(v/v) CO_2-air mixture and light intensity sufficient to allow cell division into four progeny.

Enzyme Assay

The aminating and deaminiating activity of the NADP-GDH was measured spectrophotometrically at 340 nm, by adding a 10 to 20 μL aliquot of enzyme preparation to 500 μL of assay solution. The deaminating assay solution was composed of 44 mM Tris, 20.4 mM glutamate, and 1.02 mM $NADP^+$ (Sigma), pH

8.8. The aminating assay solution was composed of 50 mM Tris, 25 mM α-ketoglutarate, 0.357 mM NADPH (Sigma), and 0.356 M $(NH_4)_2SO_4$, pH 7.4. One unit of enzyme activity was the amount of NADP-GDH required to reduce or to oxidize 1.0 μM of $NADP^+$ or NADPH per min at 38.5°C.

Isolation of RNA

All labware used in total cellular RNA isolation was sterilized by baking at 220°C for 8 h, and sterile plasticware was utilized whenever possible. All solutions were made with H_2O treated with 0.1% diethylpyrocarbonate (Sigma) overnight at 37°C, and autoclaved according to Sambrook et al. (1989).

On the day of the RNA isolation, a pellet of *C. sorokiniana* cells stored at -70°C was resuspended 1 to 10 (w/v) in RNA breakage buffer: 0.1M Tris (pH8.5), 0.4M LiCl, 10 mM EGTA, 5 mM EDTA, 100 units/mL sodium heparin (Sigma, 100 units/mg), and 1 mM aurintricarboxylic acid (Sigma). The cell suspension was centrifuged at 7000*g* for 5 min at 4°C and the supernatant was discarded. The cell pellet was resuspendeed 1 to 10 (w/v) in RNA breakage buffer and ruptured by passage through a French pressure cell at 20,000 p.s.i.. The cell homogenate was collected in a disposable 50 mL conical tube containing 0.05 times volume 20% (w/v) SDS, 0.05 times volume 0.5 M EDTA (pH 8), 200 ug/mL proteinase K (Sigma), and allowed to incubate at room temperature for 15 min. One-half volume of TE buffer (Tris 10mM:EDTA 1mM, pH

8.0) equilibrated phenol was added to the homogenate and after a 3 min incubation a one-half volume of chloroform:isoamylalcohol (24:1,v/v) was added and mixed for 10 min on a wrist action shaker (Burrel). The extracted homogenate was transferd to a 30 mL siliconized (Sigmacote, Sigma) corex tube and centrifuged at 1000*g* for 10 min at 4°C. The upper aqueous phase was removed and repeatedly extracted with an equal volume of chloroform:isoamylalcohol (24:1, v/v), as described above, until the aqueous interface was clear. After the final extraction, the aqueous phase was combined with an equal volume of 2X LiCl-Urea buffer (4 M LiCl, 4 M urea, 2 mM EDTA, 1 mM aurintricarboxylic acid) and the RNA was precipitated on ice for 16 h at 4°C. The RNA precipitate was centrifuged at 4000*g* for 20 min at 4°C and the resulting pellet was rinsed once with 1X LiCl-Urea buffer and centrifuged again to pellet the RNA. The RNA pellet was solublized in TE (pH 7.5) and an aliquot was quantified spectrophotometrically at 260 nm. After quantitation, the total RNA was precipitated with 0.3M sodium acetate (pH 5.2) and 2.5 times volume of 100% ethanol and stored at -20°C as a precipitate. The mRNA fraction was isolated from total cellular RNA using an oligo(dT) spin column kit (mRNA Separator™, Clontech) according to the supplier's instructions.

Genomic DNA Isolation

Total cellular DNA was isolated from *C. sorokiniana* cells using a modified procedure of Ausubel et al. (1989). A 6.5 g pellet of 29 mM KNO_3 cultured cells and a 6.0 g of 29 mM NH_4Cl was harvested by centrifugation at 7000 rpm for 10 min at 4°C. Each cell pellet was resuspended in 25 mL of DNA extraction buffer (0.1 M Tris-HCl, pH 8; 0.1 M EDTA, pH 8; 0.25 M NaCl), mixed with 2.7 mL of 10% (w/v) sarkosyl, and incubated for 2 h at 55°C. The cell homogenate was combined with 2.78 mL of 5M NaCl, 3.2 mL of 10% (w/v) CTAB, and incubated at 65°C for 20 min. An equal volume of chloroform:isoamylalcohol (24:1, v/v) was added and extracted by gentle shaking for 10 min followed by centifugation at 3000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and the nucleic acids were precipitated with 0.6 times volume of isopropol alcohol at -20°C for 30 min. The precipitate was pelleted by centrifugation at 4500 rpm for 20 min at 4°C. The pellet was resuspended in 9.5 ml of TE (pH 8) and combined with 10 g of CsCl (BRL) and 0.5 mL of 10 mg/mL ethidium bromide. The CsCl:DNA mix was placed on ice for 30 min and the contaminating RNA was removed by centrifugation at 7500*g* for 10 min. The CsCl supernatant was transferred to a 13 mL Quickseal tube (Beckman) and centrifuged in a Vi65 Beckman rotor for 6 h at 55,000 rpm. The high molecular weight DNA band was visualized with a hand-held UV light, eluted from the tube, butanol extracted, and dialysed 15 h against two changes of 1 L of TE (pH 8).

The dialyzed genomic DNA was quantified spectrophotometrically at 260 nm and stored as an ethanol precipitate until use.

NADP-GDH Protein Purification

Purification of the α-NADP-GDH holoenzyme

C. sorokiniana cells were cultured with continous light in 29 mM ammonium medium in a 30 L Plexiglas chamber as previously described (Baker and Schmidt, 1963). Cells were harvested at 4.0 OD_{640} by centrifugation at 30,000 rpm through a Sharples centrifuge (Pennwalt) and washed two times in 10 mM Tris (pH 8.5 at 4°C). Pelleted cells (130 g) were stored at -20°C in 250 mL centrifuge bottles until use.
Purification of NADP-GDH was accomplished using a modified procedure of Yeung et al. (1981). Procedural modifications involved the substitution of Sephadex G-200 gel (Pharmacia) for G-150 gel in the gel-filtration column, and the addition of $NADP^+$ as a stabilizer to a final concentration of 0.1 mM to the gel-filtration buffer and all subsequent storage buffers. As a final modification, the $NADP^+$ affinity resin step was omitted and a preparative nondenaturing-PAGE step was substituted (Miller et al., 1994a).

Sephadex G-200 column fractions possessing NADP-GDH activity were pooled and concentrated via Diaflow (Amicon) filtration. The soluble enzyme (68 mg) was protected from oxidation by the addition of DTT to a final concentration of

10 mM, and dialyzed for 30 min against 28.8 mM Tris, 192 mM glycine, 2 mM DTT (pH 8.4). The dialysate was clarified by centrifugation at 20,000*g* for 10 min at 4°C and was combined with 3 mL of 40% (w/v) sucrose and 1 mL of 0.02% bromophenol blue.

For preparative nondenaturing PAGE, a 3 cm tall 7% acrylamide (w/v, 28 acrylamide: 0.735 bis-acrylamide, pH 8.8) resolving gel, and a 2 cm tall 2% acrylamide (w/v, 1.6 acrylamide: 0.4 bis-acrylamide, pH 6.6) stacking gel were cast in the 28 mm ID gel tube of the Model 491 Prep Cell (Bio-Rad). All acrylamide stocks were pretreated with AG501-X8 mixed bed resin (Bio-Rad) to remove any contaminating acrylic acid residue to prevent *in vitro* N-acylation of proteins during electrophoresis. The protein sample was electrophoresed at 15 mA constant power for 20 min and then for 3.5 h at a constant power of 30 mA. Six milliliter fractions were collected and assayed for NADP-GDH deaminating activity and GDH containing fractions were pooled. The enzyme in the pooled fractions in 10 mM KPO_4 (pH 6.2), 0.1 mM $NADP^+$ was concentrated by Diaflow filtration to 1 mg/mL as determined by the method of Bradford (1976), using BSA as a standard. The concentrated enzyme preparation was stored at -20°C. The purity of the preparation was determined by silver-staining using the Silverstain Plus kit (Bio-Rad) to visualize proteins resolved by 10% (w/v) Tris-Tricine SDS-PAGE (Shagger and von Jagow, 1987).

Partial purification of NADP-GDH isoenzymes

To insure that both the α-subunit and β-subunit were represented, NADP-GDH isoenzymes were purified from a mixture of cells cultured for 240 min in 1 mM ammonium medium (14 g), 90 min in 1 mM ammonium medium (6 g), and for 20, 40, 60, and 80 min in 29 mM ammonium medium (1 g/time point) according to Bascomb and Schmidt (1987). GDH isoenzymes were partially purified using a scaled down modified procedure of Yeung et al. (1981). The DEAE sephacel ion exchange columns (pH 7.4,pH 6) were scaled down to a 40 mL bed volume and a 400 mL linear KCl gradient (0 to 0.4 M) was used to elute the proteins in 3 mL fractions. The pH 6 DEAE ion-exchange column fractions containing NADP-GDH were combined into two pools; corresponding to the leading and trailing halves of the NADP-GDH activity peak. The separate pooled fractions were dialyzed against 10 mM KPO_4 (pH 6.2), 2 mM DTT for 16 h, and affinity purified using Type 3 $NADP^+$ affinity gel (Pharmacia) as previuosly described (Bascomb and Schmidt, 1987). The NADP-GDH in the pooled fractions was concentrated via Diaflow filtration to 2 mg/ml protein, as determined by the method of Bradford (1976), and stored at 4°C until further use. After resolution of the proteins by 8% (w/v) Tris-Tricine SDS-PAGE (Shagger and von Jagow, 1987), the purity of the preparation was determined by silverstaining.

Anti-NADP-GDH Antibody Production and Purification

Monoclonal antibody production

Mouse anti-NADP-GDH MAbs were produced as described by Tamplin et al. (1991). BALB/C mice were immunized by intraperitoneal injection of 40 μg purified NADP-GDH α-subunit in 0.25 mL PBS mixed 1:1 (v/v) with Freunds complete adjuvant (Sigma). On day 24, the mice were injected intraperitonealy with 25 μg of purified α-subunit in 0.25 mL PBS mixed 1:1 (v/v) with Freunds incomplete adjuvant (Sigma). The mice were injected on day 38 with 25 μg of purified α-subunit in 0.5 mL PBS, and once again 2 days prior to cell fusion. Tail bleeds were performed one week prior to cell fusion and analysed by ELISA to select mice producing a high anti-NADP-GDH titer. Splenocytes and myeloma SP2/0 were fused by the protocol of Van Deusen and Whetstone (1981). After selection with HAT medium, hybridoma supernatants were screened by ELISA in 96 well EIA plates (Costar) against 1.25 μg NADP-GDH α-subunit in 50 μl 0.1 M Na_2CO_3 (pH 9.6) per well. The ELISA procedure was performed as previously described (Tamplin et al., 1991). ELISA positive clones were selected and hybridoma supernatants were screened for reactivity on Western blots. Selected high-titer hybridomas were cloned by limiting dilution (Harlow and Lane, 1988), and hybridoma supernatants were collected and frozen at -20°C for future use.

Polyclonal antibody production

Rabbit anti-NADP-GDH antibodies were produced commercially by Hazelton Washington (Vienna, VA) in six New Zealand white rabbits. Rabbits were given a primary immunization of 0.3 mg of purified NADP-GDH α-holoenzyme per animal; followed by mulitple 0.15 mg booster injections. Preimmune sera, test bleeds, and production bleeds were shipped on dry ice for future analyses. Anti-NADP-GDH IgG titer was determined by measuring the ability of a series of increasing amounts of antiserum or purified IgG to immunoprecipitate 1 unit of NADP-GDH activity. After incubating the immunoprecipitation mix for 35 min at 25°C, immunoprecipitates were removed by centifugation at 14,000 rpm for 2 min in an Eppendorf microfuge. The titer was recorded as the percent of NADP-GDH activity remaining in the supernatant relative to a preimmune serum control. Standard reaction conditions utilized 10 μL of 25 mM imidazole (pH 6), 1 unit of NADP-GDH deaminating activity in 20 μL of 25 mM imidazole (pH 6), anti-NADP-GDH antiserum or purified IgG in a range from 0 to 25%, and rabbit preimmune serum was added as a stabilizer to a final volume of 40 μL.

Anti-NADP-GDH IgG was purified from rabbit serum using a MASS™ Protein A affinity membrane 50 mm disc device (Nygene Corp.). Anti-NADP-GDH rabbit serum was diluted 1:4 (v/v) in 6 mL of PBS and passed through 0.45 μm syringe filter (Gelman). Filtered serum was bound to the Protein A disc at 4°C and washed with PBS until the A_{280} of the eluate was

zero. Purified IgG was eluted with 10 mL of 0.1 M glycine (pH 2.5) and 1 mL fractions were collected in microfuge tubes, containing 0.1 mL of 1 M Tris (pH 8), and then gently inverted to mix the contents. Fractions were analyzed spectrophotometrically at 280 nm and fractions containing greater than 1 mg/mL IgG were combined and frozen at -20°C in 0.25 mL aliquots.

Western Blotting

Alkaline phosphatase conjugated antibody detection

For NADP-GDH antigen determinations, the proteins in samples were resolved by 8% (w/v) Tris-Tricine SDS-PAGE using a Mini-Protean II cell (Bio-Rad) or a 400 SE cell(Hoeffer). The proteins were electroblotted to a 7 cm x 10 cm Immun-Lite™ nylon membrane (Bio-Rad) at 20 V constant voltage for 16 to 20 h at 4°C in a Mini-Transblot II cell (Bio-Rad). Whatmann 3MM filter paper, scotch-brite pads, and Immun-Lite™ membrane, utilized in the electrotransfer, were pre-equilibrated for 10 min in 10 mM MES (pH 6), 0.01% (w/v) SDS transfer buffer at room temperature. The immunodetection procedure was performed at room temperature using the Immun-Lite™ chemoluminescent detection kit (Bio-Rad). The nylon membrane was incubated for 1 h in TBS blocking buffer (20 mM Tris, 0.5 M NaCl, pH 7.5; 5% [w/v] nonfat dry milk). The membrane was washed for 5 min with TTBS (TBS with 0.05% [v/v] Tween 20) and incubated 2 to 16 h in primary antibody buffer

(TTBS, 1% [w/v] nonfat dry milk, rabbit anti-NADP-GDH IgG [1.9 mg/mL IgG faction] diluted 1:200 or mouse anti-NADP-GDH MAb hybridoma supernatant diluted 1:10). The nylon membrane was washed for 15 min with three changes of 40 mL TTBS, and incubated for 1 h in secondary antibody buffer(TTBS, 1% [w/v] nonfat dry milk, 1:3,000 dilution of goat anti-rabbit IgG AP conjugate [Bio-Rad] or 1:1,500 dilution of goat anti-mouse IgG [whole molecule] AP conjugate [Sigma]). The immunoblot was washed for 15 min with 3 changes of 40 mL TTBS, and 5 min in 40 mL of TBS. The membrane was immersed for 5 min in chemoluminescent substrate, sealed in a heat-sealable bag (Seal-n-Save, Sears), and exposed to Kodak X-Omat AR film for 30 s to 5 min. After chemoluminescent detection, the immonoblot was rinsed for 5 min in TBS, and incubated with a color development substrate (10 mL 0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 50 mM MgCl, 45 μL NBT, 35 μL X-phosphate [Boehringer Manniheim]). Color development was monitored visually and stopped by the addition of 30 mL of TE (pH 8), and molecular weight determinations were made relative to a series of prestained protein markers (Midrange kit, Diversified Biotech; Rainbow markers, Amersham).

^{125}I-Protein A detection

The proteins in 12 μL aliquots of clarified cell homogenates (20 mL cell culture concentrated in 3 mL of GDH breakage buffer) were resolved by 8% (w/v) Tris-Tricine SDS-PAGE in the 400 SE cell. The gel was electrophoresed at 10

mA constant current until the prestain 30 kD protein marker (Rainbow markers, Amersham) reached the bottom of the gel. The SDS gel, nitrocellulose (Bioblot-NC, Costar), and Whatmann 3MM filter paper were equilibrated for 30 min in 24.8 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.4 transfer buffer (Towbin et al. 1979). The proteins were transferred electrophoretically at 30 V constant voltage for 16 h at 4°C in a Trans-Blot cell (Bio-Rad). Immunodetection was performed using a modified procedure of Towbin and Gordon (1984) and Johnson et al. (1984). After electroblotting, the nitrocellulose was air-dried and blocked in 50 mL of 40 mM Tris (pH 7.4), 150 mM NaCl, 5% (w/v) nonfat dry milk (Carnation), and 0.01% (v/v) Antifoam A (Dow-Corning) blocking buffer for 1 h. All incubations were performed at room temperature in a heat-sealable bag on a Labquake shaker (Lab Industries). The blocking solution was decanted and the nitrocellulose was incubated in 30 mL of 40 mM Tris (pH 7.4), 150 mM NaCl, 5% (w/v) nonfat dry milk, 0.01% (v/v) Antifoam A, 0.05% (v/v) Tween 20 (Batteiger et al., 1982), and rabbit anti-NADP-GDH antibody (1.9 mg/mL, IgG fraction) diluted 1:300 for 3 h. The immunoblot washed for 1 h with three changes of 200 mL of 40 mM Tris (pH 7.4), 150 mM NaCl and transferred to 25 mL of 40 mM Tris, (pH 7.4), 150 mM NaCl, 5% (v/v) nonfat dry milk, 0.01% (v/v) Antifoam A, 0.05% (v/v) Tween 20, and 0.25 μCi/lane of ^{125}I-labelled Protein A (30 mCi/mg, Amersham) for 1 h in a new heat-sealable bag. The nitrocellulose was washed for 1 h with three changes of 300

mL of 40 mM Tris (pH 7.4), 150 mM NaCl. The immunoblot was allowed to air dry for 3 h on a stack of paper towels and exposed to Fuji RX autoradiogrphy film at -70°C.

Amino-Terminal Sequence Analysis of the NADP-GDH α-Subunit and β-Subunit

An aliquot of a preparation of purified NADP-GDH α-subunit (120 pmol) and a partially purified preparation of NADP-GDH α-subunit (80 pmol) and β-subunit (50 pmol) were resolved by 8% (w/v) Tris-Tricine SDS-PAGE and electroblotted to a PVDF membrane (Immobilon-P^{SQ}, Millipore) as described by Plough et al. (1989). To prevent *in vitro* acylation of the protein amino-terminal residues, all polyacrylamide solutions used in PAGE were treated with AG501-X8 mixed bed resin to remove contaminating acrylic acid. Protein sequence analysis of the electroblotted proteins was provided by the ICBR Protein Chemistry Core facility.

DNA Probe Synthesis

Specific cDNA restriction fragments were excised from purified plasmids using the appropriate restriction endonuclease (BRL). The plasmid restriction endonuclease fragments were separated by electrophoresis in an alkaline agarose gel (0.8% to 3% [w/v]) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The ethidium bromide stained fragment of interest was cut out of the gel and mascerated in the upper portion of a 0.45 μm nylon membrane microfilterfuge

tube (Ranin). The mascerated agarose was frozen for 1 h at -20°C and then centrifuged at 3000 rpm in an Eppendorf microfuge. The gel purified fragment in the supernatant was precipitated with 0.3 M sodium acetate (pH 5.2) and 2.5 times volume of 100% ethanol at -20°C.

Twenty-five nanograms of purified cDNA fragment were diluted in 33 μL dH_20 and denatured at 100°C and chilled rapidly on ice. The denatured fragment was radiolabeled with ^{32}P-dGTP (3000 Ci/mmol, Amersham) by the random primer method of Feinberg and Vogelstein (1984). Unicorporated nucleotides were removed from the ^{32}P-labeled probe using a Sehadex G-50 (Pharmacia) spin column (Sambrook et al., 1989).

Northern Blot Analysis

Total or poly(A)$^+$ RNA stored as an ethanol precipitate was pelleted by centrifugation at 14000 rpm for 15 min in an Eppendorf microfuge. The vacuum dried pellet was resuspended in 20 μL of 75% (v/v) formamide (BRL), 8.28% (v/v) formaldehyde, 3% (v/v) 10X MOPS (20 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7) and heated for 5 min at 80°C then chilled on ice. The denatured RNA sample was combined with 6 μL of formamide loading buffer (Sambrook et al., 1989) and 1 μL of 10 mg/mL ethidium bromide and resolved on a 2% (w/v) formaldehyde-agarose gel (Ausubel et al., 1989). The RNA was electrophoresed at 5 V/cm with constant circulation of the electrophoresis buffer using magnetic stir bars in the buffer reservoirs. After visualization with a UV transilluminator

(Fotodyne), the RNA was transferred by capillary blotting to a Hybond-N nylon membrane (Amersham) with 20X SSPE (3.6 M NaCl, 0.2 M KPO_4, pH 7.7; 20 mM EDTA). The nylon membrane was rinsed once in 2X SSPE, air dried for 1 h, and UV irradiated for 5 min on a transilluminator to covalently link the RNA to the membrane.

The membrane was prehybridized for 2 h at 40°C in 10 mL of 50% (v/v) formamide, 25 mM KPO_4 (pH 7.7), 5X SSPE, 5X Denhardt,s solution (0.1% [w/v] Ficoll, 0.1% [w/v] polyvinylpyrrolidone, 0.1% [w/v] BSA), 0.1% (w/v) SDS, 100 μg/mL sheared denatured salmon sperm DNA, 100 μg/mL yeast tRNA in a heat-sealable bag on a Labquake shaker. The prehybrization solution was decanted and the membrane was hybridized for 16 h at 42°C on a Labquake shaker in 10 mL of prehybridization buffer with 1 x 10^6 to 1 x 10^8 cpm of a heat denatured ^{32}P-labeled cDNA probe. The membrane was washed three times in 0.1X SSPE, 0.1% (w/v) SDS for 20 min per wash at 65°C. The Northern blot was covered with laboratory plastic wrap and exposed to Kodak X-Omat AR film with one intensifying sceen at -70°C.

Southern Blot Analysis

C. sorokiniana high molecular weight genomic DNA (6 μg) was digested with a threefold excess of *Pvu* II, or *Taq* I (BRL) for 4 h at the appropriate buffer and temperature conditions. Three, 2 μg aliquots of *Pvu* II digested, *Taq* I digested, and undigested genomic DNA were electrophoresed at

1.5 V/cm in a 0.8% (w/v) agarose gel in TAE buffer. Ethidium bromide (0.5 μg/mL) was added to the gel prior to polymerization to allow visualization of the DNA during electrophoresis. After electrophoresis, the gel was soaked for 10 min in 0.1 N HCl, 35 min in 0.5 N NaOH, 1.5 M NaCl denaturing solution, and 45 min in 0.5 M Tris, 3 M NaCl, pH 7 neutralizing solution. The DNA was transferred to a Hybond-N nylon membrane by capillary action using 20X SSC (3 M NaCl, 0.3M sodium citrate) as described by Southern (1975). After transfer, the membrane was rinsed in 2X SSC, air dried, and the DNA was covalently linked to the membrane for 5 min on a UV transilluminator. The nylon membrane was cut into three strips each containing *Pvu* II digested, *Taq* I digested, and uncut genomic DNA for analysis with different probes.

The nylon membranes were prehybridized in a HB-1D hybridization oven (Techne) in 15 mL of 20% (v/v) formamide, 0.6 M NaCl, 0.6 M sodium citrate, 10 mM EDTA, 0.1% (w/v) SDS, 5X Denhardt's solution, 100 μg/mL denatured sheared salmon sperm DNA for 2 h at 40°C. The membranes were hybridized independently for 16 h at 45°C in 15 mL of 50% (v/v) formamide, 10% (w/v) dextran sulfate (Sigma), 1X Denhardt's solution, 4X SSC, 5 μg/mL denatured sheared salmon sperm DNA, and 1 x 10^6 to 1 x 10^8 cpm denatured ^{32}P-labeled cDNA probe. The cDNA probes corresponded to the 5'-VR, HCR, or 3'-UTR of the concensus *C. sorokiniana* NADP-GDH mRNAs. The membranes were washed independently three times in 50 mL of 0.1X SSC, 0.1% (w/v) SDS for 30 min per wash at 65°C. The nylon

membranes were covered with plastic wrap and exposed to Fuji RX film with one intensifying screen at -70°C.

NADP-GDH cDNA Cloning and Characterization

λgt10 library

Small scale liquid lysates of previously isolated plaque pure NADP-GDH λgt10 clones were produced as described by Sambrook et al. (1989). The λgt10 clone DNA was isolated using a rapid small scale liquid lysate λ phage DNA isolation procedure (Ausubel et al., 1989). The cDNA inserts were excised from the purified phage DNA with *Eco* RI restriction endonuclease, gel purified as described above, subcloned into the multiple cloning site of pUC 18, and transformed by the $CaCl_2$ method (Ausubel et al., 1989) into *Eschericia coli* DH5α for further characterization.

λZAP II library

Synchronous *C. sorokiniana* cells were cultured in a 3 L chamber in 1 mM ammonium medium or 29 mM ammonium medium according to conditions reported by Bascomb and Schmidt (1987) to yield primarily the NADP-GDH α-holoenzyme or predominately the β-holoenzyme, respectively. The level of ammonia in the 1 mM ammonium medium induction was periodically measured by the method of Hardwood and Kuhn (1970). Cells were harvested by centrifugation at 8000*g* at 4°C, and frozen at -70°C for use in total RNA isolation. The

cells in 20 mL from each growth condition were concentrated by filtration, resuspended in 3 mL of GDH breakage buffer, and ruptured by passage through a French pressure cell at 20,000 p.s.i.. Aliquots of the various homogenates from ammonium induced cells were resolved by 7.5% (w/v) nondenaturing PAGE and the NADP-GDH isoenzyme pattern was determined by use of a selective activity stain (Yeung et al., 1981).

Total cellular RNA was isolated from 2 g cell pellets from each growth condition as described above and the poly(A)$^+$ RNA fraction was purified by elution from an oligo(dT) cellulose spin column (Clontech). The poly(A)$^+$ selection was repeated two times on each RNA preparation to insure complete removal of contaminating tRNAs, and rRNAs. Both total RNA (20 μg) and poly(A)$^+$ RNA (10 μg) were analyzed by 2% (w/v) formaldehyde-agarose gel electrophoresis, and northern blot analysis, using a 242 bp HCR radiolabeled cDNA probe, to verify the purity, intactness, and approximate quantity of NADP-GDH mRNA represented in the α-induced and β-induced RNA preparations. Poly(A)$^+$ RNA (50 μg) from each preparation was combined and utilized for the commercial production of a custom λUni-ZAP XR *C. sorokiniana* cDNA library (Stratagene Cloning Systems, Palo Alto, CA).

The amplified λZAP library, containing 2 x 10^{10} pfu/mL, was plated on twenty 150 mm petri plates at 50,000 pfu per plate for a total of 1 x 10^6 pfu screened. The phage plaques were absorbed to duplicate Hybond-N 132 mm circular membranes

and treated according to the plaque blotting protocol of Amersham (1985). Membranes were prehybridized in a common container in 200 mL of 2X PIPES (0.8 M NaCl, 20 mM PIPES, pH 6.5), 50% (w/v) formamide, 0.5% (w/v) SDS, 100 μg/mL denatured sheared salmon sperm DNA at 40°C. Blocked membranes were hybridized at 42°C in ten heat-sealable bags (four membranes/bag) in prehybridization buffer containing 1 x 10^6 cpm/membrane of a ^{32}P-labeled NADP-GDH 242 bp HCR cDNA probe on a lab rocker (Reliable Scientific). The membranes were washed three times in 200 mL of 0.1X SSC, 0.1% (w/v) SDS for 20 min per wash at 50°C. Duplicate membranes were wrapped in plastic wrap and exposed to Kodak X-Omat AR film at -70°C for 28 h. Putative NADP-GDH cDNA plagues, detected on duplicate membranes, were cored from the plate and plaque purified by secondary and tertiary screenings with the 242 bp HCR probe. Putative NADP-GDH cDNA phage clones (167), selected in the primary screening, were combined and screened a second time with a ^{32}P-labeled 130 bp *Eco* RI/*Bgl* II cDNA fragment isolated from the 5' terminus of the most complete 5' end NADP-GDH cDNA clone (pGDc 42). Ten plaque pure NADP-GDH clones were subcloned in pBluescript KS$^+$ and transformed into *E. coli* DH5α F' via an *in vivo* excision protocol provided by Stratagene. All plasmid isolations were performed as described by Kraft et al. (1988).

5' RACE-PCR cloning

The 5'-terminal NADP-GDH cDNA sequences were cloned using a modified anchored PCR procedure for the rapid amplification of cDNA ends (Frohman, 1990; Jain et al., 1992). A mixture of poly(A)$^+$ RNA, used in the synthesis of the λZAP library, was utilized to clone the 5' end of the NADP-GDH mRNA. One hundred nanograms of the mRNA mixture were combined with 10 ng of a gene-specific primer (RRS 9; Table 1), designed to hybridize to the HCR of NADP-GDH mRNAs, heated for 5 min, and chilled on ice. First strand DNA synthesis was performed using Superscript™ reverse transcriptase (BRL) according to the supplier's protocol. The terminated reverse transcription reaction was treated with one unit of ribonuclease H for 20 min at 37°C, 5 min at 95°C, and extracted once with chloroform:isoamyl alcohol (24:1, v/v). Excess primers and dNTPs were removed by centrifugation at 2000 rpm through an Ultrafree-MC filterfuge tube (30,000 MW cutoff, Millepore) and the retentate was concentrated to 10 μl on a Savant Speedvac. The first-strand synthesis products were combined with 10 μL of tailing mix (1X tailing buffer [Promega Corp.], 0.4 mM dATP, 10 units terminal deoxytransferase) and incubated at 37°C for 10 min. The reaction mixture was heated to 95°C for 5 min, diluted to 0.5 mL with TE (pH 8), and utilized as a cDNA pool. A mixture of 5μL of the cDNA pool, 5 μL of Vent™ polymerase 10X buffer (NEB), 200 μM of each dNTP, 25 pmol of a gene specific

Table 1. Synthetic oligonucleotide sequences

Oligomer	Nucleotide Sequence
RRS5	GGGCTGCGCAGGCCGGGCGGCCACGATAGG
RRS6	GGGTCGACATTCTAGACAGAATTCGTGGATCC$(T)_{18}$
RRS7	GGGTCGACATTCTAGACAGAA
RRS9	CTCAAAGGCAAGGAACTTCATG
RRS11	GGACGAGTACTGCACGC
RRS12	GAGCAGATCTTCAAGAACAGC
RRS13	TCTGCACGTAGCTGATGTGG
RRS14	CCCAGCCAGGGCCCTCACC
RRS15	CACAGTATCGCATTCCGGGC
RRS16	GATCTCGGTCAGCAGCTG
RRS17	CTTTCTGCTCGCCCTCTC
RRS18	GCGGCGACATCGCGC
RRS19	CGTGCGCCAGCTGCTGAC
RRS24	CCTTGTTGTACTTGTGG
RRS25	CCACAAGTACAACAAGG

primer (RRS 9), 5 pmol of the poly(dT) adaptor primer (RRS6), 0.2 units Perfectmatch™ DNA polymerase enhancer (Stratagene), and 1 unit of Vent™ polymerase (NEB) in 50 μL was amplified according to Jain et al. (1992). The PCR products were purified away from the excess primers by centrifugation at 2,000 rpm through an Ultrafree-MC unit. The retentate was collected and subjected to two more rounds of amplification using a new nested gene specific primer at each step (RRS 11; RRS16, respectively) and an adaptor primer (RRS 7). PCR amplifications were performed in a Model 480 thermocycler (Perkin-Elmer Cetus), and all custom oligonucleotides were synthesized by the ICBR DNA synthesis facility. The standard PCR reaction mixture consisted of 10 μL of 10X Vent™ polymerase buffer, 100 μM of each dNTP, 0.4 units of Perfectmatch™, 50 pmol of each primer, 1 unit Vent™ DNA polymerase in a 100 μl reaction volume. The optimal PCR cycling parameters were determined using Oligo™ 4.0 primer analysis software (National Biosciences Inc.). The 5' RACE-PCR products were gel purified, subcloned into the *Sma* I site of pUC 18, and transformed into *E. coli* DH5α for further characterization.

NADP-GDH cDNA characterization

Purified NADP-GDH cDNA clone plasmids were digested for 1 to 2 h with specific restriction endonucleases using buffer and temperature conditions deemed optimal by the supplier (BRL). The resulting DNA fragments were resolved by

electrophoresis in a 1% (w/v) agarose minigel in TAE buffer. DNA fragments less than 500 bp were resolved by 4% (w/v, 29 acrylamide:1 bis-acrylamide) PAGE in TBE buffer (0.13 M Tris, 45 mM Borate, 2.5 mM EDTA, pH 9) at 7 V/cm until the bromophenol blue dye was 1.5 cm from the base of the gel. Restriction fragment size was determined by comparison to λDNA/*Hind* III and ΦX174/*Hae* III restriction fragment standards (BRL).

NADP-GDH cDNA clones were sequenced by the dideoxy method of Sanger et al. (1977) using modified T7 polymerse (Tabor and Richardson, 1987) and the Sequenase 2.0 kit protocol (United States Biochemical Corp.). The products of the sequencing reactions were resolved on a 7 M urea, 5% (w/v, 29 acrylamide:1 bis-acrylamide) sequencing gel. All cDNA clones were partially sequenced from both ends. Internal sequences were determined by subcloning select restriction fragments into pUC 18 or by generation of a set of nested deletions by timed digestion with exonuclease III (Henikoff, 1984) using the Erase-a-base system (Promega Corp.). Sequence data were analyzed using the Genetics Computer Group programs (Devereux et al., 1984) on the ICBR VAX computer.

Primer Extension Analysis

The 5' transcriptional start sites of the NADP-GDH mRNAs were mapped by primer extension analysis as described by Sambrook et al. (1989). A 20 μg aliqout of a poly(A)$^+$ RNA

mixture, isolated for use in the λZAP library synthesis, was combined with 3.5 x 105 cpm of a ^{32}P-labeled 30 nucleotide oligomer (RRS 5) designed to hybridize to the 5' end of the NADP-GDH mRNA. The primer:RNA mixture was denatured at 85°C for 10 min and allowed to anneal for 12 h at 30°C. After annealing, the RNA:primer complex was extended at 45°C for 2 h with 5 units of Superscript™ reverse transcriptase, 0.5 mM of each dNTP, according to the supplier's protocol. The extension reaction products were treated with 5 ng of DNase free RNase A (Sigma) for 30 min at 37°C, phenol:chloroform:isoamylalcohol (25:24:1, v/v) extracted, and ethanol precipitated at 0°C for 1 h. The pelleted precipitate was resuspended in 6 μL of formamide loading buffer and resolved on a 7 M urea, 5% (w/v) acrylamide sequencing gel. The primer extension product was detected by autoradiography on Kodak X-Omat AR film at -70°C and the size of the extension product was estimated by comparison to a sequencing ladder.

Genomic Allele-Specific PCR

C. sorokiniana genomic DNA was analysed by allele-specific PCR as described by Saiki et al. (1986). Genomic DNA (1 μg) and three NADP-GDH genomic clones in pUC 18 (0.1 ng; pGDg 8.4.4, 14.10.1, 15.2.2) were amplified using exon-specific primer pairs that hybridized to exons one and three (RRS17, RRS18), exons 10 and 11 (RRS12, RRS13), and exon 22 (RRS14, RRS15) of the NADP-GDH gene. The standard genomic

PCR reaction mixture was composed of 1X Vent™ polymerase buffer, 200mM of each dNTP, 50 pmol of each primer, 0.4 units Perfectmatch™, and 1 unit Vent™ polymerase. PCR cycles were executed under cycling parameters deemed optimal for each primer pair. The allele-specific PCR products were resolved by 4% (w/v) PAGE and visualized by ethidium staining. The size of the PCR products was estimated relative to a 123 bp ladder (BRL).

Construction of NADP-GDH *In vitro* Transcription Vectors

PCR generated fragments corresponding to the 5'-VR of the +42 nt and -42 nt mRNAs, HCR, and 3'-UTR were cloned downstream of the SP6 promoter of the SP65 *in vitro* transcription vector (Promega Corp.). Using the 5' RACE-PCR +42 bp and -42 bp cDNA clones (5'-VR; RRS16, RRS17) or pGDc 23 (HCR; RRS 12, RRS13: 3'-UTR; RRS14, RRS15) as templates, the PCR fragments were amplified, gel purified, and cloned into the *Sma*I site of pUC 18. Clones corresponding to each of the mRNA regions were selected on the basis of their orientation in pUC 18, excised by digestion with *Sal* I/*Eco* RI, and directionally cloned in the antisense orientation into the SP65 transcription vector. Constructs were verified by sequence analysis and were purified by a large scale CsCl plasmid isolation procedure (Ausubel et al., 1989). ^{32}P-labeled antisense RNA probes corresponding to the four regions of the mRNA were transcribed using the Riboprobe™ *in vitro* transcription system (Promega Corp.). Full length *in*

vitro transcription products were selected by gel purification on a 7 M urea, 5% (w/v) acrylamide sequencing gel according to Ausubel et al. (1989) and used as protecting fragments for RPA.

Comparison of the NADP-GDH mRNAs, Antigens, and Activities in 29 mM Induced *C. sorokiniana* Cells

Culture conditions

C. sorokiniana cells were synchronized using three alternating light:dark periods (9 h: 7 h) in 29 mM KNO_3 medium. Cells were harvested by centrifugation, washed with nitrogen-free medium, and resuspended in 4 L of pre-equilibrated nitrogen-free medium in a 4 L Plexiglas chamber. The cells were induced in 29 mM ammonium medium for 240 min as described by Cock et al. (1991). Samples of 500 mL of cell culture (2.87×10^6 cells/mL)were collected at T_0, and at 20 min intervals for the first 140 min, and a final sample was harvested at 240 min. Samples for RNA isolation were concentrated by centrifugation at 4°C at 9,000 rpm and stored at -70°C. The cells in 20 mL of culture were harvested by filtration, resuspended in 3 mL of GDH breakage buffer, and stored at -20°C for protein and GDH activity analyses.

RNase protection analysis

Total cellular RNA was isolated from a 1 g pellet from each time point as described above. The amount of poly(A)+ RNA in the various total RNA preparations was quantified based on the formation of ribonuclease-resistant hybrids with poly-^{3}H-5,6-uridylate (2-10 Ci/mmol, New England Nuclear) as described by Davis and Davis (1978) using purified β-globin mRNA (BRL) as a standard.

The quanity and form of mRNA present at each time interval was determined by RPA. A total of 210 ng of Poly (A)+ RNA or 25 ng of control yeast tRNA was combined with 1 x 10^5 cpm of a ^{32}P-labeled antisense RNA probe corresponding to the +42 nt and -42 nt 5'-VR, HCR, or 3'-UTR of the NADP-GDH mRNAs and hybridized for 16 h at 42°C. RPAs were performed using the Guardian™ RPA kit (Clontech) according to the suppliers instructions. The ribonuclease resistant fragments were resolved on a 7 M urea, 5% (w/v) sequencing gel and sized by comparison to a sequencing ladder. The resistent fragments were transferred from the gel to 3MM Whatmann filter paper, dried at 80°C, and exposed to a phosphorous screen and then to Fuji RX film at -70°C. The amount of residual antisense RNA probe was quantitated on a PhosphoImager™ (Molecular Dynamics) at the ICBR DNA Synthesis Core facility.

NADP-GDH antigen and activity analyses

C. sorokiniana cell samples from the various time points were disrupted by two passages through a French pressure cell at 20,000 p.s.i.. Aliquots from each cell homogenate were analyzed for both aminating and deaminating NADP-GDH activity. Total soluble protein concentration in the cell homogenates was determined by the method of Bradford (1976) using BSA as a standard. The proteins in 12 μL aliquots from each homogenate were resolved by 8% (w/v) Tris-Tricine SDS-PAGE, transferred to nitrocellulose, and NADP-GDH antigen was detected by ^{125}I-Protein A Western blot analysis as described above. The amount of NADP-GDH antigen present as the α-subunit and β-subunit at each time interval was quantified on a Visage 60 laser desitometer (Bio Image).

RT-PCR analysis

A modified RT-PCR (Kawaski, 1990) procedure was used to identify and quantify the NADP-GDH mRNAs present in the total RNA preparations from the various induction time-points. A series of primer pairs were selected to yield ovelapping PCR fragments spanning the entire length of the NADP-GDH mRNAs: RRS17, RRS18; RRS17, RRS16; RRS19, RRS13; RRS12, RRS13; RRS12, RRS24; RRS15, RRS25. An aliquot of total RNA from each time-point containing 10 μg of poly(A)$^+$ RNA was combined with 300 pmol of random hexameric oligonucleotides (Pharmacia), incubated at 75°C for 5 min, and chilled on ice. The RNA:primer mixture in 6 μL was combined with 2 μL of 10X

Vent™ polymerase buffer, 10 units RNASIN™ (Promega Corp.), 1 mM of each dNTP, 1 mM DTT, 200 units Superscript™ reverse transcriptase in a final volume of 20 μL, and incubated at 22°C for 10 min, 42°C for 70 min, 50°C for 30 min, and 95°C for 5 min. After termination of the reaction, 120 μL of 1X Vent™ polymerase buffer was added to each reaction tube. The resulting mixture served as cDNA stocks for subsequent amplifications. The standard RT-PCR amplification mixture consisted of 8 μL 10X Vent™ polymerase buffer, 200 μM of each dNTP, 50 pmol of each primer, 0.2 units Perfectmatch™, 20 μL of a cDNA stock, and 4 units of Vent™ (exo-) polymerase (NEB) in 100 μL final volume. The RT-PCR mixtures were cycled at conditions determined to be optimal for each primer pair using the Oligo™ 4.0 primer analysis software. RT-PCR products were resolved on 1% to 3% (w/v) agarose gels and sized by comparison to a 123 bp ladder (BRL). The relative intensities of the PCR fragments was quantified from Polaroid™ Type 55 negatives of ethidium bromide stained gels on a Visage 60 laser densitometer.

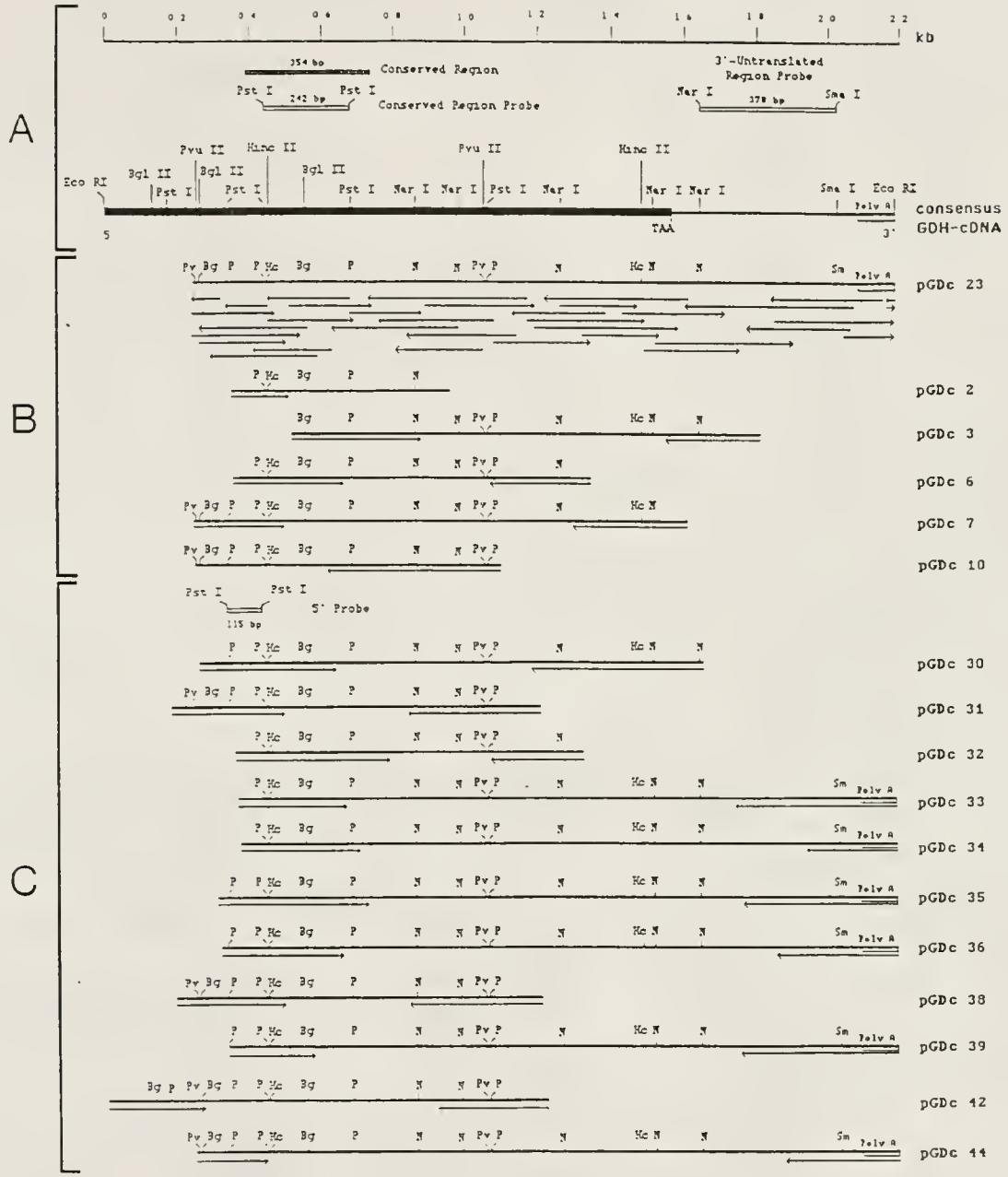
RESULTS

NADP-GDH cDNA Cloning and Characterization

Restriction mapping and sequencing of λgt10 cDNA clones

A λgt10 cDNA library was constructed from poly(A)$^+$ RNA isolated from *C. sorokiniana* cells induced in 29 mM ammonium medium for 80 min. Cells induced under these conditions were reported by Prunkard et al. (1986) and Bascomb et al. (1987) to accumulate both the α- and β-subunits as NADP-GDH holoenzymes between 40 and 120 min, and at 80 min the α- and β-subunits each constituted approximately 50% of the total NADP-GDH antigen. Approximately 2 x 10^6 pfu were screened with a heterologous 1.2 kb *Salmonella typhimurium gdhA* gene probe (Miller and Brenchley, 1984) and six putative NADP-GDH cDNAs were isolated. The cDNAs ranged from 0.6 to 1.91 kb and their restriction maps were identical in regions in which they overlapped (Fig. 1B; Cock et al., 1991). The cDNA clones appeared to be truncated forms of the 1.91 kb pGDc 23 cDNA clone that lacked a complete 5' terminus. A *lacZ*-pGDc 23 translation fusion expressed in *E. coli* JM 109 accumulated antigen which was recognized by antibodies raised to purified *C. sorokiniana* NADP-GDH verifying the cDNA authenticity (Cock et al., 1991).

Figure 1. Restriction maps of 17 cDNAs isolated from a *C.sorokinana* library prepared from RNA isolated from cells induced for 80 min in 29 mM ammonium medium. A, The 2145 bp consensus NADP-GDH map. Regions corresponding to the HCR and 3'-UTR are indicated. B, NADP-GDH cDNA clones isolated using a heterologous 1.2 kb probe from the *gdhA* gene from *S. typhymurium*. cDNA clones were sequenced in the regions denoted by arrows. C, The cDNA clones were isolated using a homologous 115 bp *Pst*I fragment from the 5' end of the HCR of pGDc 23.


A
B
C
kb
Conserved Region
Conserved Region Probe
3'-Untranslated Region Probe
Pst I
Nar I
Sma I
Pvu II
Hinc II
Bgl II
Eco RI
consensus
GDH-cDNA
TAA
pGDc 23
pGDc 2
pGDc 3
pGDc 6
pGDc 7
pGDc 10
5' Probe
pGDc 30
pGDc 31
pGDc 32
pGDc 33
pGDc 34
pGDc 35
pGDc 36
pGDc 38
pGDc 39
pGDc 42
pGDc 44

In an attempt to select additional NADP-GDH cDNAs with complete 5' termini, the λgt10 library was rescreened with a homologous 115 bp *Pst*I restriction fragment probe (Fig. 1C) derived from the 5'-proximal end of pGDc 23. Approximately one-half of the 115 bp probe sequence overlaps the 5-terminus of the 354 bp NADP-GDH HCR (Fig. 1A) that is predicted to be present in all NADP-GDH cDNAs. Eleven additional cDNA clones were isolated, restriction-mapped, and their 3' and 5' termini sequenced (Fig. 1C). The additional cDNA clones had identical restriction maps and sequences in regions that ovelapped with pGDC 23. NADP-GDH cDNA clones pGDC 31, 38, and 42 were longer than pGDC 23 at their 5' termini; however, none of the cDNAs possessed an ATG start methionine codon or a 5'-untranslated region. The 5'-terminal *Eco*RI/*Pvu*II fragment (Fig. 1C) of pGDC 42 was subcloned into pUC 18 and both strands were sequenced. Sequence analysis of the pGDc 42 subclone revealed that pGDc 42 possesed an additional 256 bp of coding region sequence not found in pGDc 23.

Although the cDNA library was prepared using an oligo (dT) primer, 10 of the 17 NADP-GDH cDNAs lacked a poly(A)$^+$ tail and additional 3'-terminal sequences (Fig. 1). None of the 17 cDNA clones were full-length at their 5' termini. From the combined sequences of the cDNA clones, a 2145 bp consensus NADP-GDH cDNA sequence and restricition map was constructed (Fig.1A). Bascomb et al. (1986) and Cock et al. (1991) showed the NADP-GDH mRNA to be 2.2 kb; therefore, the

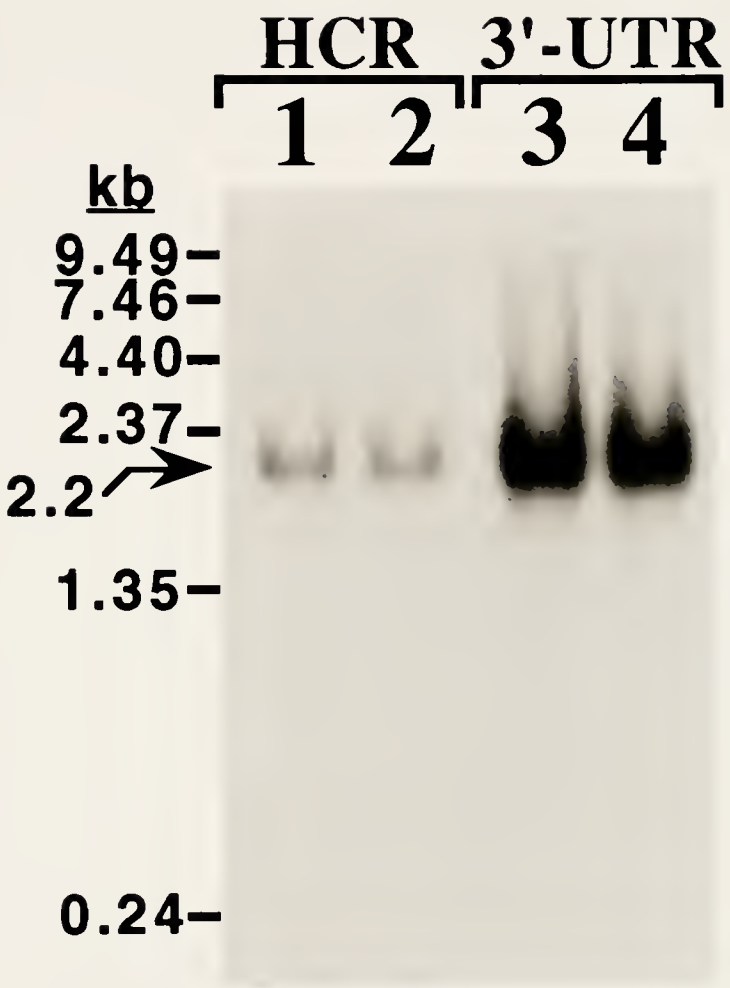
consensus NADP-GDH clone was determined to be approximately 98% full-length.

Isolation, restriction-mapping, and sequencing of the λZAPII NADP-GDH cDNA clones

Since all of the λgt10 library NADP-GDH cDNA clones lacked complete 5' termini and many lacked complete 3' termini, a second ammonium-induced unidirectional cDNA library was constructed. Synchronous *C. sorokiniana* cells, used for total RNA isolation, were cultured in 1 mM or 29 mM ammonium medium according to conditions reported to yield primarily the NADP-GDH α-holoenzyme or predominately the β-holoenzyme, respectively (Bascomb and Schmidt, 1987). The NADP-GDH isoenzyme pattern from each culture condition was verified by activity staining crude preparations resolved by nondenaturing PAGE. Poly(A)$^+$ RNA from each culture condition was analyzed by northern analysis, using the 242 bp HCR and the 378 bp 3'-UTR probes (Fig. 1A), to verify the approximate level and intactness of NADP-GDH mRNA in each preparation (Fig. 2). An equal quantity of poly(A)$^+$ RNA from each preparation was combined, to ensure representation of multiple types of NADP-GDH mRNAs if they existed, and the RNA mixture was utilized in the cDNA library construction. The λZAPII unidirectional cloning system was utilized to ensure the cloning of the complete 3' ends of the mRNAs.

Initial screening of 1 x 10^6 pfu with the homologous 242 bp HCR probe derived from pGDc 23 (Fig. 1A) yielded 167

Figure 2. Northern blot analysis of poly(A)$^+$ RNA isolated from *C. sorokiniana* cells induced for 3 h in 1 mM ammonium medium (Lanes 1,3) or continuously in 29 mM ammonium medium (Lanes 2,4). Formaldehyde-agarose gel resolved RNA preparations immobilized on nylon were hybridized with the 242 bp HCR probe and 378 bp 3'-UTR probe. A single-sized 2.2 kb NADP-GDH mRNA was detected in both RNA preparations with both probes.


HCR
3'-UTR
1
2
3
4
kb
9.49
7.46
4.40
2.37
2.2
1.35
0.24

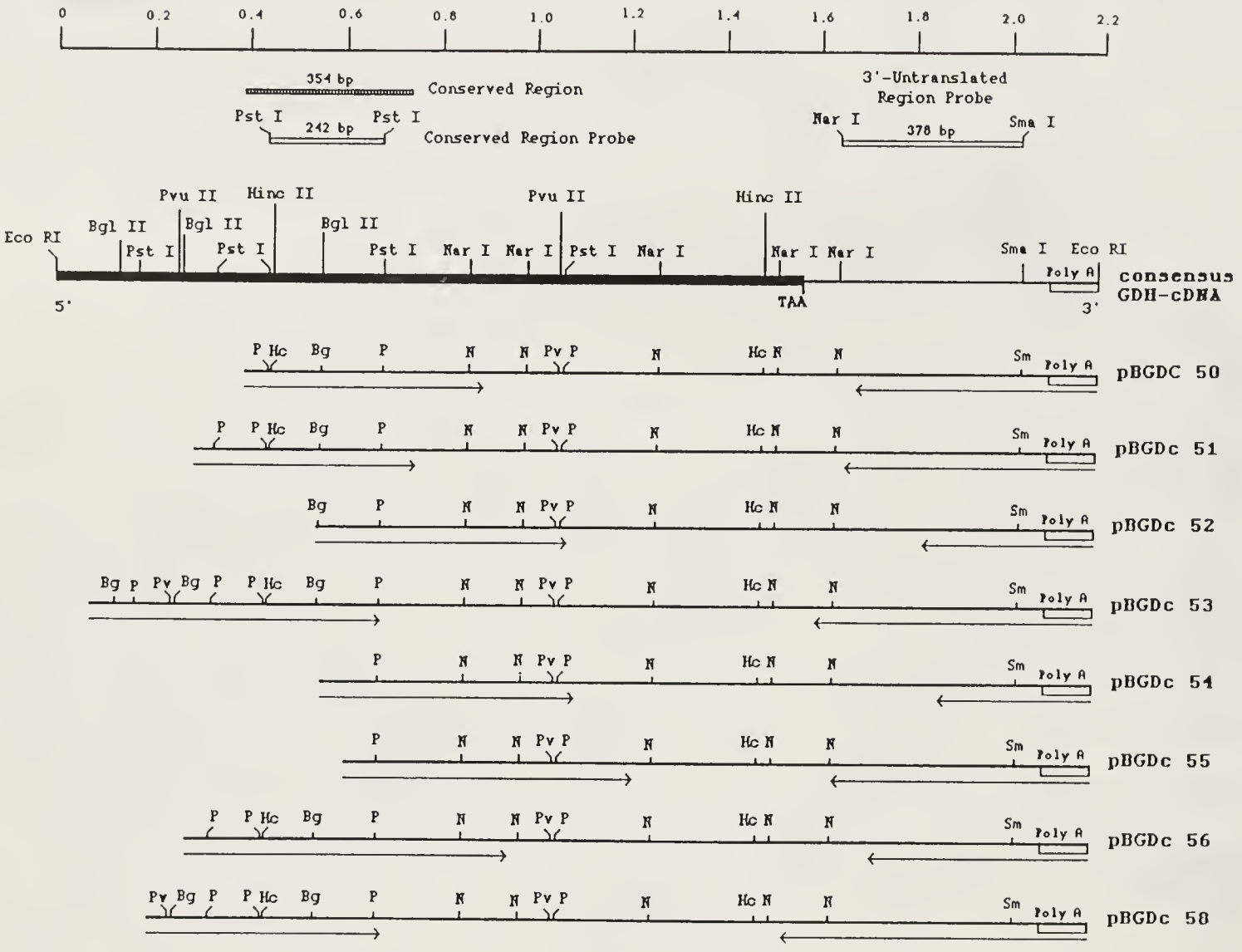
putative NADP-GDH cDNA clones. Ten of the primary screening plaques were selected at random and plaque purified by secondary and tertiary screenings. Restriction-mapping and sequencing of the ten NADP-GDH cDNAs revealed eight unique overlapping clones ranging in size from 1.46 to 2.04 kb (Fig. 3). Two of the 10 clones proved to be identical to pBGDC 52 and 58 indicating a detection of redundant clones in the amplified library. All eight of the unique NADP-GDH clones possessed identical complete 3' termini; however, they all lacked complete 5' termini (Fig. 3). The longest λZAP NADP-GDH cDNA clone, pBGDc 53, was 103 bp shorter than the λgt10 NADP-GDH consensus cDNA.

To detect any cDNA clones longer at the 5' terminus than pBGDc 53, the 167 putative NADP-GDH plaques selected in the primary screening were combined and rescreened. The second screening utilized a homologous 130 bp *Eco*RI/*Bgl*II cDNA fragment probe derived from the 5' terminus of pGDc 42 (Fig. 1C). No NADP-GDH clones longer than pBGDc 53 were isolated from the secondary screening.

Primer extension analysis

Although all of the NADP-GDH cDNA clones isolated from the λZAP library possessed complete 3' ends, none of the 25 cDNA clones isolated from either library possessed complete 5' ends. In order to determine the amount of sequence remaining proximal to the 5' end of the consensus NADP-GDH cDNA clone, primer extension analysis was performed on the

Figure 3. Restriction maps of eight cDNAs isolated from a *C. sorokiniana* library prepared from a RNA mixture containing equal amounts of poly(A)$^+$ RNA from cells induced in 1 mM ammonium and 29 mM ammonium medium. NADP-GDH clones were isolated using a homologous 242 bp HCR probe derived from pGDC 23 (Fig. 1). All clones possess a complete 3' terminus and no clones were longer than the previously determined consensus NADP-GDH cDNA.


0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
354 bp
Conserved Region
Pst I
242 bp
Pst I
Conserved Region Probe
3'-Untranslated
Region Probe
Nar I
378 bp
Sma I
Eco RI
Bgl II
Pst I
Pvu II
Hinc II
Nar I
Sma I
Eco RI
Poly A
consensus
GDH-cDNA
5'
TAA
3'
pBGDC 50
pBGDc 51
pBGDc 52
pBGDc 53
pBGDc 54
pBGDc 55
pBGDc 56
pBGDc 58

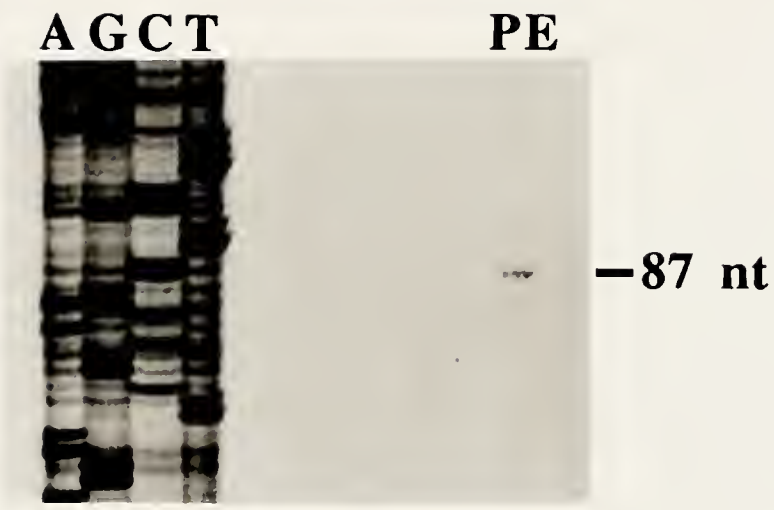
mixture of *C. sorokiniana* poly(A)$^+$ RNA previously isolated for the λZAP library construction. As determined by comparison to a cDNA generated sequencing ladder, a single primer extension product of 87 nt was detected (Fig. 4). The 87 nt product corresponds to 53 bp of sequence identified in the 5' terminus of pGDc 42 (Fig. 1C) and 34 bp of sequence previously undetected. The additional 34 bp extension predicted a mRNA of 2.179 kb that approximated the 2.2 kb mRNA determined by northern blot analysis (Fig. 2). The primer extension analysis was repeated with identical results.

RACE-PCR cloning of two NADP-GDH 5' termini

To determine the 5'-terminal sequences of the NADP-GDH mRNA(s), a modified anchored PCR procedure for the rapid amplification of cDNA ends was performed (Jain et al., 1992). To ensure any possible sequence differences that might reside in the 5' region proximal to the HCR would not be missed, a RNA mixture previously prepared by mixing equal quantities of poly(A)$^+$ RNA from cells synthesizing primarily the α- or β-holoenzyme was used for the RACE-PCR cloning.

Agarose gel elelctrophoresis of the products from the second step of the RACE-PCR amplification revealed two DNA fragments of approximately 390 and 450 bp in size. Reamplification using a different nested gene-specific primer, designed to hybridize closer to the 5'-termini of the mRNAs, yielded two unique PCR products of approximately 330

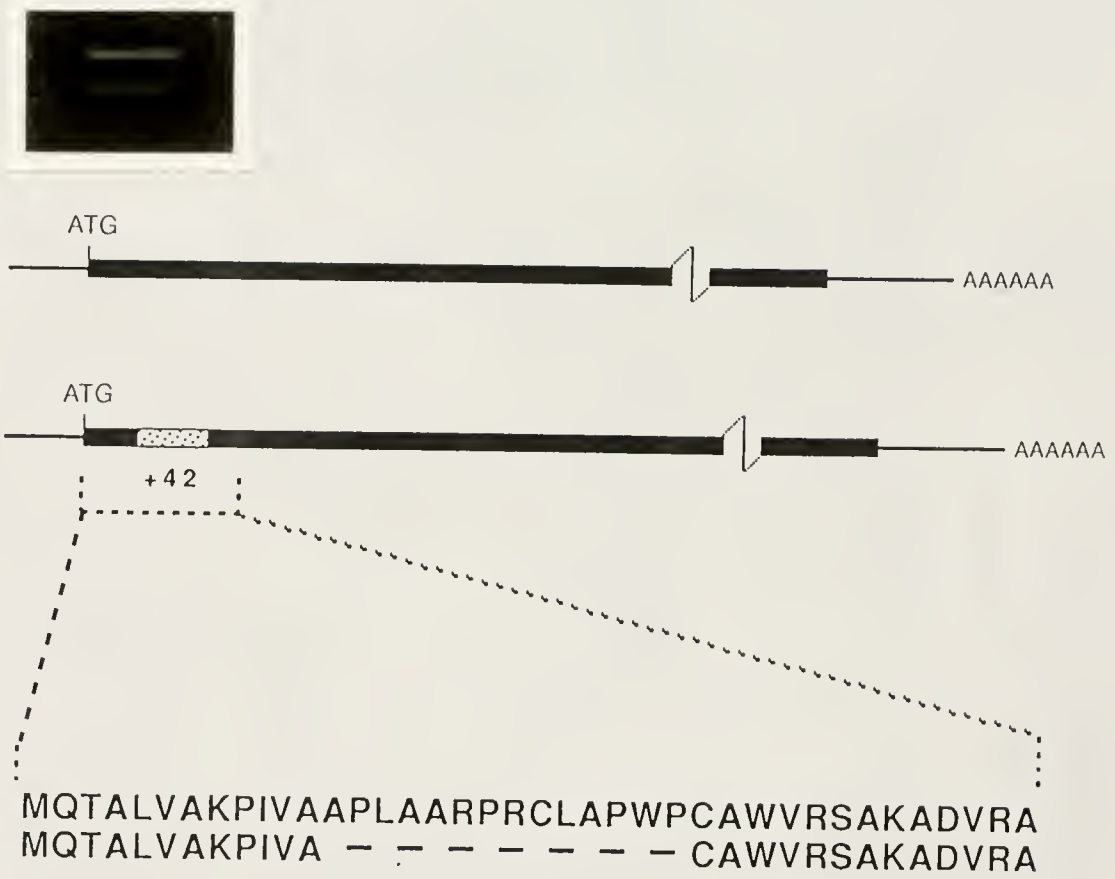
Figure 4. Primer extension analysis of NADP-GDH mRNA(s). A poly(A)$^+$ RNA mixture isolated from *Chlorella* cells, synthesizing primarily the NADP-GDH α- or β-subunit, was hybridized with a ^{32}P-labelled 5' NADP-GDH-specific oligonucleotide and extended with reverse transcriptase. A single 87 nt primer extension product (PE) was detected after resolution on a 5% sequenceing gel. The approximate size of the extension product was determined by comparison to a NADP-GDH cDNA clone sequencing ladder.


A G C T
P E
—87 nt

and 370 bp in size as determined by agarose gel electrophoresis (Fig. 5). Sequence analysis of the two cloned final PCR products revealed the actual size of the products, minus the anchor primer, to be 269 and 311 bp. Comparison of the two RACE-PCR cDNA clone sequences showed them to be identical except for the presence of an additional 42 bp in the 5' coding region of the longer PCR product. The additional 42 bp sequence encodes 14 amino acids that were not present in the 269 bp RACE-PCR clone (Fig. 5). The absence of the 42 bp sequence in the 269 bp clone results in the deletion of the 14 amino acids from the amino-terminus of the polypeptide; however, the downstream reading frame remained unchanged. Both RACE-PCR products possessed identical putative 5'-UTRs, translation initiation sites, and were identical for the first 12 codons (Fig. 5). Both the +42 bp and -42 bp clones overlapped in frame with pGDC 42 at their 3' ends. The 5' RACE products were 33 bp longer than pGDC 42 at their 5'-termini which is in close agreement with the 34 bp length predicted by primer extension analysis.

The NADP-GDH 5' RACE-PCR clones possessed identical 32 bp 5'-terminal pyrimidine rich (89%) sequences and transcription initiation sequences indicative of eukaryotic 5'-UTRs and translational start sites, respectively (Kozak, 1984). Furthermore, additional 5'-terminal guanine residues were detected (2 on the +42 bp and 1 on the -42 bp clone) in both clones that could not be accounted for in the NADP-GDH gene sequence. The presence of unique 5'-terminal guanine

Figure 5. 5' RACE-PCR generated NADP-GDH 5'-terminus clones. Two PCR products, approximately 370 and 330 bp, were detected after resolution on a 3% agarose gel (inset). Sequence analysis revealed the two 5'-end clones differed by 42 bp in their 5'-coding regions. The deduced amino acid sequences of the two NADP-GDH mRNAs showed the two mRNAs to encode identical polypeptides with the exception of an additional 14 amino acids encoded by the 42 nt insert in the longer mRNA.


ATG
AAAAAA
ATG
AAAAAA
+42
MQTALVAKPIVAAPLAARPRCLAPWPCAWVRSAKADVRA
MQTALVAKPIVA – – – – – – – CAWVRSAKADVRA

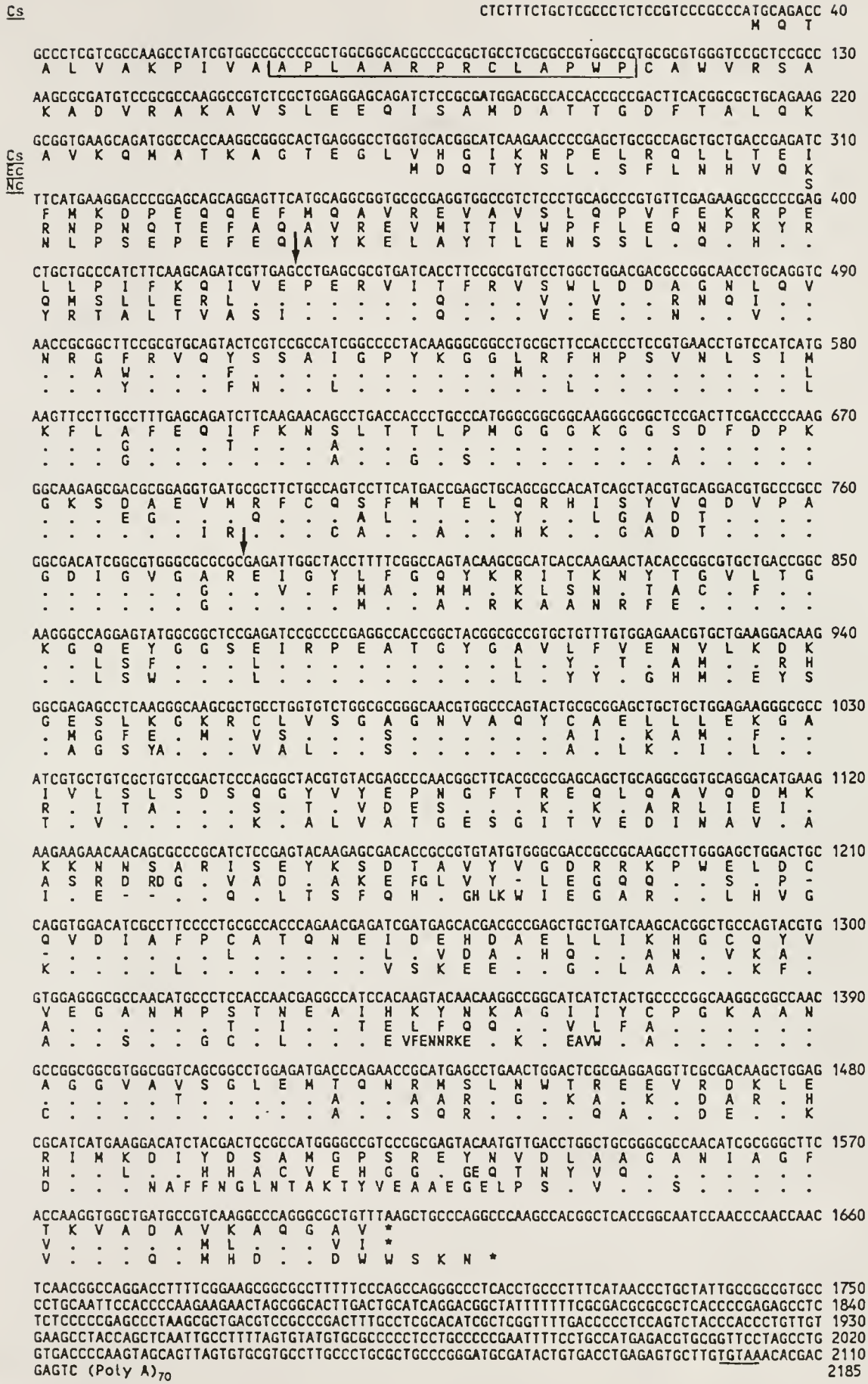
residues on 5' RACE-PCR products has proven to be a definitive means of identifying 5' capping points and transcription initation sites of eukaryotic mRNAs (Bahring et al., 1994). The RACE-PCR procedure was repeated with identical results. RT-PCR performed on the poly(A)$^+$ RNA mixture using two new gene-specific primers that flanked the 42 bp variable region also yielded two PCR products differing in size by 42 bp.

Both 5' RACE-PCR products appear to be complete at their 5' termini and overlap in frame with the consensus NADP-GDH cDNA identified earlier. These results are consistent with the existence of two separate NADP-GDH mRNAs that share a common transcriptional start site and are identical with the exception of a 42 nt insert identified in the longer mRNA. The +42 nt mRNA predicted size is 2185 nt, whereas the -42 nt mRNA predicted size is 2143 assuming a mean poly(A) tail length of 70 nt.

Analysis of the *C. sorokiniana* NADP-GDH cDNA sequences

Sequence analysis of the two consensus NADP-GDH cDNAs (+42 bp and -42 bp) revealed both mRNAs possessed an identical 32 nt 5'-UTR (Fig 6). The +42 bp cDNA possesses an ORF from nt 33 to a TAA stop codon at nt 1611 that encodes a precursor protein with a molecular mass of 57850 D. The -42 bp cDNA possesses an ORF from nt 33 to nt 1569 that encodes a precursor protein of 56350 D. The 1500 D difference in molecular mass observed in the two precursor proteins is due

Figure 6. Nucleotide sequence of the consensus NADP-GDH mRNAs derived from the cDNA and 5' RACE-PCR clone sequences. Beginning at nucleotide 33, two ORFs were identified from two mRNAs that differed by 42 nt in the 5'-VR (boxed). The +42 bp mRNA encodes a polypeptide of 57,850 D, whereas the -42 bp mRNA encodes a 56,350 D polypeptide. The deduced amino acid sequences of the *C. sorokiniana* precursor polypeptides (Cs) are compared with those of *E. coli* (Ec) and *N. crassa* (Nc) NADP-GDHs. Arrows denote the boundaries of the highly conserved glutamate binding domain identified by Mattaj et al. (1982). A consensus algal polyadenylation signal (underlined) is located 17 bp upstream from the poly(A) tail of the NADP-GDH mRNAs.


Cs
CTCTTTCTGCTCGCCCTCTCCGTCCCGCCCATGCAGACC 40
M Q T
GCCCTCGTCGCCAAGCCTATCGTGGCCGCCCCGCTGGCGGCACGCCCCGCGCTGCCTCGCGCCGTGGCCGTGCGCGTGGGTCCGCTCCGCC 130
A L V A K P I V A A P L A A R P R C L A P W P C A W V R S A
AAGCGCGATGTCCGCGCCAAGGCCGTCTCGCTGGAGGAGCAGATCTCCGCGATGGACGCCACCACCGCCGACTTCACGGCGCTGCAGAAG 220
K A D V R A K A V S L E E Q I S A M D A T T G D F T A L Q K
GCGGTGAAGCAGATGGCCACCAAGGCGGGCACTGAGGGCCTGGTGCACGGCATCAAGAACCCCGAGCTGCGCCAGCTGCTGACCGAGATC 310
A V K Q M A T K A G T E G L V H G I K N P E L R Q L L T E I
M D Q T Y S L . S F L N H V Q K
S
TTCATGAAGGACCCGGAGCAGCAGGAGTTCATGCAGGCGGTGCGCGAGGTGGCCGTCTCCCTGCAGCCCGTGTTCGAGAAGCGCCCCGAG 400
F M K D P E Q Q E F M Q A V R E V A V S L Q P V F E K R P E
R N P N Q T E F A Q A V R E V M T T L W P F L E Q N P K Y R
N L P S E P E F E Q A Y K E L A Y T L E N S S L . Q . H . .
CTGCTGCCCATCTTCAAGCAGATCGTTGAGCCTGAGCGCGTGATCACCTTCCGCGTGTCCTGGCTGGACGACGCCGGCAACCTGCAGGTC 490
L L P I F K Q I V E P E R V I T F R V S W L D D A G N L Q V
Q M S L L E R L . . . . . Q . . . V . V . . R N Q I . .
Y R T A L T V A S I . . . . . Q . . . V . E . . N . . V . .
AACCGCGGCTTCCGCGTGCAGTACTCGTCCGCCATCGGCCCCTACAAGGGCGGCCTGCGCTTCCACCCCTCCGTGAACCTGTCCATCATG 580
N R G F R V Q Y S S A I G P Y K G G L R F H P S V N L S I M
. . A W . . . F . . . . . M L
. . . Y . . . F N . . . L . . . . . L L
AAGTTCCTTGCCTTTGAGCAGATCTTCAAGAACAGCCTGACCACCCTGCCCATGGGCGGCGGCAAGGGCGGCTCCGACTTCGACCCCAAG 670
K F L A F E Q I F K N S L T T L P M G G G K G G S D F D P K
. . . G . . . T . . . A . . . . . .
. . . G . . . . . A . . G . S . . . . . A . . . . .
GGCAAGAGCGACGCGGAGGTGATGCGCTTCTGCCAGTCCTTCATGACCGAGCTGCAGCGCCACATCAGCTACGTGCAGGACGTGCCCGCC 760
G K S D A E V M R F C Q S F M T E L Q R H I S Y V Q D V P A
. . . E G . . . Q . . . A L . . . . Y . . L G A D T . . . .
. . . . . I R . . . C A . . A . . H K . . G A D T . . . .
GGCGACATCGGCGTGGGCGCGCGCGAGATTGGCTACCTTTTCGGCCAGTACAAGCGCATCACCAAGAACTACACCGGCGTGCTGACCGGC 850
G D I G V G A R E I G Y L F G Q Y K R I T K N Y T G V L T G
. . . . . G . . V . F M A . M M . K L S N . T A C . F . .
. . . . . G . . . . . M . . A . R K A A N R F E . . . . .
AAGGGCCAGGAGTATGGCGGCTCCGAGATCCGCCCCGAGGCCACCGGCTACGGCGCCGTGCTGTTTGTGGAGAACGTGCTGAAGGACAAG 940
K G Q E Y G G S E I R P E A T G Y G A V L F V E N V L K D K
. . L S F . . . L . . . . . L . Y . T . A M . . R H
. . L S W . . . L . . . . . L . Y Y . G H M . E Y S
GGCGAGAGCCTCAAGGGCAAGCGCTGCCTGGTGTCTGGCGCGGGCAACGTGGCCCAGTACTGCGCGGAGCTGCTGCTGGAGAAGGGCGCC 1030
G E S L K G K R C L V S G A G N V A Q Y C A E L L L E K G A
. M G F E . M . V S . . . S . . . . . A I . K A M . F . .
. A G S YA . . . V A L . . S . . . . . A . L K . I . L . .
ATCGTGCTGTCGCTGTCCGACTCCCAGGGCTACGTGTACGAGCCCAACGGCTTCACGCGCGAGCAGCTGCAGGCGGTGCAGGACATGAAG 1120
I V L S L S D S Q G Y V Y E P N G F T R E Q L Q A V Q D M K
R . I T A . . . S . T . V D E S . . . K . K . A R L I E I .
T . V . . . . . K . A L V A T G E S G I T V E D I N A V . A
AAGAAGAACAACAGCGCCCGCATCTCCGAGTACAAGAGCGACACCGCCGTGTATGTGGGCGACCGCCGCAAGCCTTGGGAGCTGGACTGC 1210
K K N N S A R I S E Y K S D T A V Y V G D R R K P W E L D C
A S R D RD G . V A D . A K E FG L V Y - L E G Q Q . . S . P -
I . E - - . . Q . L T S F Q H . GH LK W I E G A R . . L H V G
CAGGTGGACATCGCCTTCCCCTGCGCCACCCAGAACGAGATCGATGAGCACGACGCCGAGCTGCTGATCAAGCACGGCTGCCAGTACGTG 1300
Q V D I A F P C A T Q N E I D E H D A E L L I K H G C Q Y V
- . . . . . L . . . . . L . V D A . H Q . . A N . V K A .
K . . . . L . . . . . V S K E E . . G . L A A . . K F .
GTGGAGGGCGCCAACATGCCCTCCACCAACGAGGCCATCCACAAGTACAACAAGGCCGGCATCATCTACTGCCCCGGCAAGGCGGCCAAC 1390
V E G A N M P S T N E A I H K Y N K A G I I Y C P G K A A N
A . . . . . T . I . . T E L F Q Q . . V L F A . . . . . .
A . . S . . G C . L . . . E VFENNRKE . K . EAVW . A . . . . . .
GCCGGCGGCGTGGCGGTCAGCGGCCTGGAGATGACCCAGAACCGCATGAGCCTGAACTGGACTCGCGAGGAGGTTCGCGACAAGCTGGAG 1480
A G G V A V S G L E M T Q N R M S L N W T R E E V R D K L E
. . . . . T . . . . . A . . A A R . G . K A . K . D A R . H
C . . . . . A . . S Q R . . . . Q A . . D E . . K
CGCATCATGAAGGACATCTACGACTCCGCCATGGGGCCGTCCCGCGAGTACAATGTTGACCTGGCTGCGGGCGCCAACATCGCGGGCTTC 1570
R I M K D I Y D S A M G P S R E Y N V D L A A G A N I A G F
H . . L . . H H A C V E H G G . GE Q T N Y V Q . . . . . .
D . . . N A F F N G L N T A K T Y V E A A E G E L P S . V . . S . . . . .
ACCAAGGTGGCTGATGCCGTCAAGGCCCAGGGCGCTGTTTAAGCTGCCCAGGCCCAAGCCACGGCTCACCGGCAATCCAACCCAACCAAC 1660
T K V A D A V K A Q G A V *
V . . . . . M L . . . V I *
V . . . Q . M H D . . D W W S K N *
TCAACGGCCAGGACCTTTTCGGAAGCGGCGCCTTTTTCCCAGCCAGGGCCCTCACCTGCCCTTTCATAACCCTGCTATTGCCGCCGTGCC 1750
CCTGCAATTCCACCCCAAGAAGAACTAGCGGCACTTGACTGCATCAGGACGGCTATTTTTTTCGCGACGCGCGCTCACCCCGAGAGCCTC 1840
TCTCCCCCGAGCCCTAAGCGCTGACGTCCGCCCGACTTTGCCTCGCACATCGCTCGGTTTTGACCCCCTCCAGTCTACCCACCCTGTTGT 1930
GAAGCCTACCAGCTCAATTGCCTTTTAGTGTATGTGCGCCCCCTCCTGCCCCCGAATTTTCCTGCCATGAGACGTGCGGTTCCTAGCCTG 2020
GTGACCCCAAGTAGCAGTTAGTGTGCGTGCCTTGCCCTGCGCTGCCCGGGATGCGATACTGTGACCTGAGAGTGCTTGTGTAAACACGAC 2110
GAGTC (Poly A)70 2185

to the presence of the additional 14 amino acid residues in the +42 bp cDNA (Fig. 6).

The sequence TGTAA located 17 nt upstream of the polyadenylation site has been identified as a conserved polyadenylation signal generally used in algal genomes (Fig. 6). The conserved TGTAA signal has been identified in the same position in numerous *Chlamydomonas*, *Chlorella*, *Volvox*, and *Euglena* cDNAs (Wolf et al., 1991).

The deduced amino acid sequences of the *C. sorokiniana* NADP-GDHs are 50% and 50.3% identical with the NADP-specific GDH of *E. coli* (McPherson and Wooton, 1983) and *Neurospora crassa* (Kinnaird and Finchum, 1983), respectively (Fig. 6). Comparison of the sequences of the highly conserved glutamate binding domain (Mattaj et al., 1982) shows a strong identity of 76.6% and 73.4%, respectively. Alignment of the *C. sorokiniana* NADP-GDH polypeptide sequences with the bovine mitochondrial NAD-dependent GDH (Julliard and Smith, 1979) revealed a significantly lower 23% identity for the entire protein and a 27.4% identity over the GDH conserved region.

Analysis of the codon preference of both NADP-GDH mRNAs showed a strong bias for C and G at the first position, in the case of arginine and leucine, and the third position of the codon (Table 2, Table 3). Furthermore, an extreme preference for G or C at the third codon position correlates with the 63% GC content reported for the *C. sorokiniana* genomic DNA (Cock et al., 1990).

Table 2. Codon usage of the -42 bp NADP-GDH mRNA.

TTT	phe	F	2	TCT	ser	S	1	TAT	tyr	Y	2
TTC	phe	F	17	TCC	ser	S	16	TAC	tyr	Y	16
TTA	leu	L	-	TCA	ser	S	-	TAA	OCH	Z	1
TTG	leu	L	-	TCG	ser	S	3	TAG	AMB	Z	-
CTT	leu	L	2	CCT	pro	P	3	CAT	his	H	-
CTC	leu	L	2	CCC	pro	P	14	CAC	his	H	6
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	-
CTG	leu	L	32	CCG	pro	P	3	CAG	gln	Q	28
ATT	ile	I	1	ACT	thr	T	2	AAT	asn	N	1
ATC	ile	I	25	ACC	thr	T	18	AAC	asn	N	19
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	-
ATG	met	M	15	ACG	thr	T	2	AAG	lys	K	37
GTT	val	V	4	GCT	ala	A	3	GAT	asp	D	3
GTC	val	V	8	GCC	ala	A	31	GAC	asp	D	23
GTA	val	V	-	GCA	ala	A	-	GAA	glu	E	-
GTG	val	V	31	GCG	ala	A	15	GAG	glu	E	35

TGT	cys	C	-	CGT	arg	R	-	AGT	ser	S	-
TGC	cys	C	8	CGC	arg	R	26	AGC	ser	S	8
TGA	OPA	Z	-	CGA	arg	R	-	AGA	arg	R	1
TGG	trp	W	4	CGG	arg	R	-	AGG	arg	R	-
TGT	cys	C	-	CGT	arg	R	-	AGT	ser	S	-
TGC	cys	C	8	CGC	arg	R	26	AGC	ser	S	8
TGA	OPA	Z	-	CGA	arg	R	-	AGA	arg	R	1
TGG	trp	W	4	CGG	arg	R	-	AGG	arg	R	-
TGT	cys	C	-	CGT	arg	R	-	AGT	ser	S	-
TGC	cys	C	8	CGC	arg	R	26	AGC	ser	S	8
TGA	OPA	Z	-	CGA	arg	R	-	AGA	arg	R	1
TGG	trp	W	4	CGG	arg	R	-	AGG	arg	R	-
TGT	cys	C	-	CGT	arg	R	-	AGT	ser	S	-
TGC	cys	C	8	CGC	arg	R	26	AGC	ser	S	8
TGA	OPA	Z	-	CGA	arg	R	-	AGA	arg	R	1
TGG	trp	W	4	CGG	arg	R	-	AGG	arg	R	-

Table 3. Codon usage of the +42 bp NADP-GDH mRNA.

TTT	phe	F	2	TCT	ser	S	1	TAT	tyr	Y	2
TTC	phe	F	17	TCC	ser	S	16	TAC	tyr	Y	16
TTA	leu	L	-	TCA	ser	S	-	TAA	OCH	Z	1
TTG	leu	L	-	TCG	ser	S	3	TAG	AMB	Z	-
CTT	leu	L	2	CCT	pro	P	3	CAT	his	H	-
CTC	leu	L	3	CCC	pro	P	14	CAC	his	H	6
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	-
CTG	leu	L	33	CCG	pro	P	7	CAG	gln	Q	28
ATT	ile	I	1	ACT	thr	T	2	AAT	asn	N	1
ATC	ile	I	25	ACC	thr	T	18	AAC	asn	N	19
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	-
ATG	met	M	15	ACG	thr	T	2	AAG	lys	K	37
GTT	val	V	4	GCT	ala	A	3	GAT	asp	D	3
GTC	val	V	8	GCC	ala	A	32	GAC	asp	D	23
GTA	val	V	-	GCA	ala	A	1	GAA	glu	E	-
GTG	val	V	31	GCG	ala	A	17	GAG	glu	E	35

TGT	cys	C	-	CGT	arg	R	-	AGT	ser	S	-
TGC	cys	C	9	CGC	arg	R	28	AGC	ser	S	8
TGA	OPA	Z	-	CGA	arg	R	-	AGA	arg	R	1
TGG	trp	W	5	CGG	arg	R	-	AGG	arg	R	-
TGT	cys	C	-	CGT	arg	R	-	AGT	ser	S	-
TGC	cys	C	9	CGC	arg	R	28	AGC	ser	S	8
TGA	OPA	Z	-	CGA	arg	R	-	AGA	arg	R	1
TGG	trp	W	5	CGG	arg	R	-	AGG	arg	R	-
TGT	cys	C	-	CGT	arg	R	-	AGT	ser	S	-
TGC	cys	C	9	CGC	arg	R	28	AGC	ser	S	8
TGA	OPA	Z	-	CGA	arg	R	-	AGA	arg	R	1
TGG	trp	W	5	CGG	arg	R	-	AGG	arg	R	-
TGT	cys	C	-	CGT	arg	R	-	AGT	ser	S	-
TGC	cys	C	9	CGC	arg	R	28	AGC	ser	S	8
TGA	OPA	Z	-	CGA	arg	R	-	AGA	arg	R	1
TGG	trp	W	5	CGG	arg	R	-	AGG	arg	R	-

Both of the deduced NADP-GDH polypeptides possess amino-terminal extensions that are rich in alanine, serine, and threonine and contain few acidic residues. These amino acid sequence motifs are indicative of chloroplast targeting domains (Smeekens et al., 1990). The boundaries of the transit peptide were delineated by amino terminal sequence analysis as discussed later.

The secondary structures of the deduced polypeptide sequences of the +42 nt and -42 nt GDH cDNAs were predicted by the method of Garnier et al. (1978; Fig. 7, Fig. 8). Alignment of the predicted secondary structures to homologous regions of the *Clostridium symbiosum* NAD-GDH 1.96Å resolution crystal structure (Baker et al., 1992) indicates the predicted structures are accurate representations. Comparison of the chloroplast transit peptide regions of the two predicted structures showed that the 14 amino acids encoded by the additional 42 nt of the longer mRNA introduced a random coil structure with multiple turns (Fig. 8) that disrupts an α-helical domain observed in the -42 nt mRNA transit peptide region (Fig. 7).

Determination of the Exon/Intron Boundaries of the NADP-GDH Gene

A 9873 bp region of genomic DNA containing the NADP-GDH gene previously sequenced by Cock et al. (1991) was compared to the two consensus NADP-GDH cDNA sequences (Fig. 9). Both the +42 bp and -42 bp NADP-GDH cDNAs span a 7178 bp region of

Figure 7. Secondary structure prediction of the *C. sorokiniana* -42 bp NADP-GDH mRNA precursor polypeptide. Structural predictions were made according to Garnier et al. (1978) and were verified by comparison to the secondary structures determined from the three-dimensional crystal structure of the *C. symbiosum* NAD-GDH. Arrow denotes region of α-helicies that is disrupted by the additional 14 amino acids in the +42 bp mRNA precursor polypeptide (Fig. 8).

α-helix
β-sheet
Turn
NH2
HOOC
50
100
200
250
300
350
400
450
500

Figure 8. Secondary structure prediction of the *C. sorokiniana* +42 bp NADP-GDH mRNA precursor polypeptide. Structural predictions were made according to Garnier et al. (1978) and were verified by comparison to the secondary structures determined from the three-dimensional crystal structure of the *C. symbiosum* NAD-GDH. Arrows denote borders of region of secondary structure introduced by the additional 14 amino acid residues unique to the +42 bp NADP-GDH mRNA.

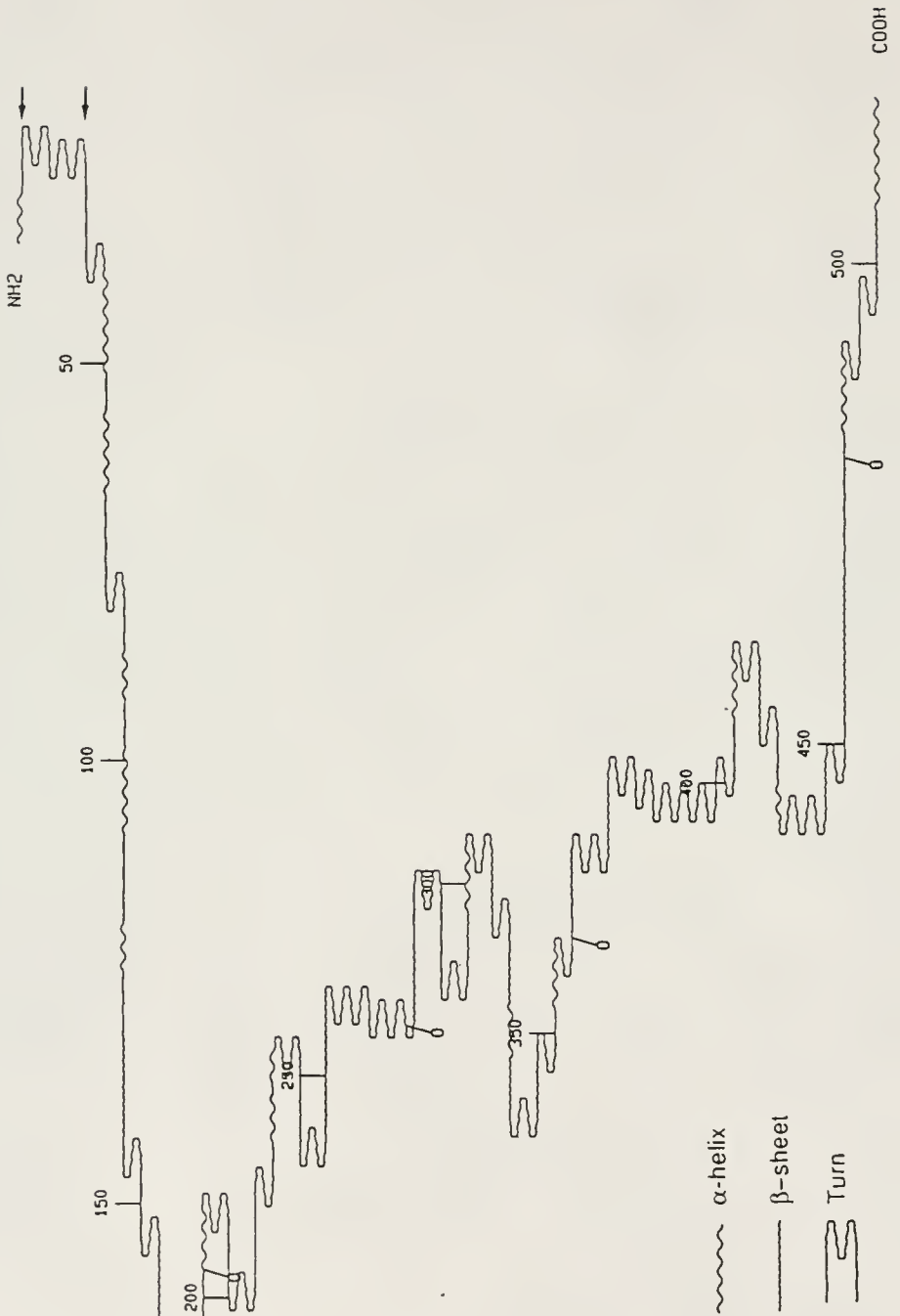
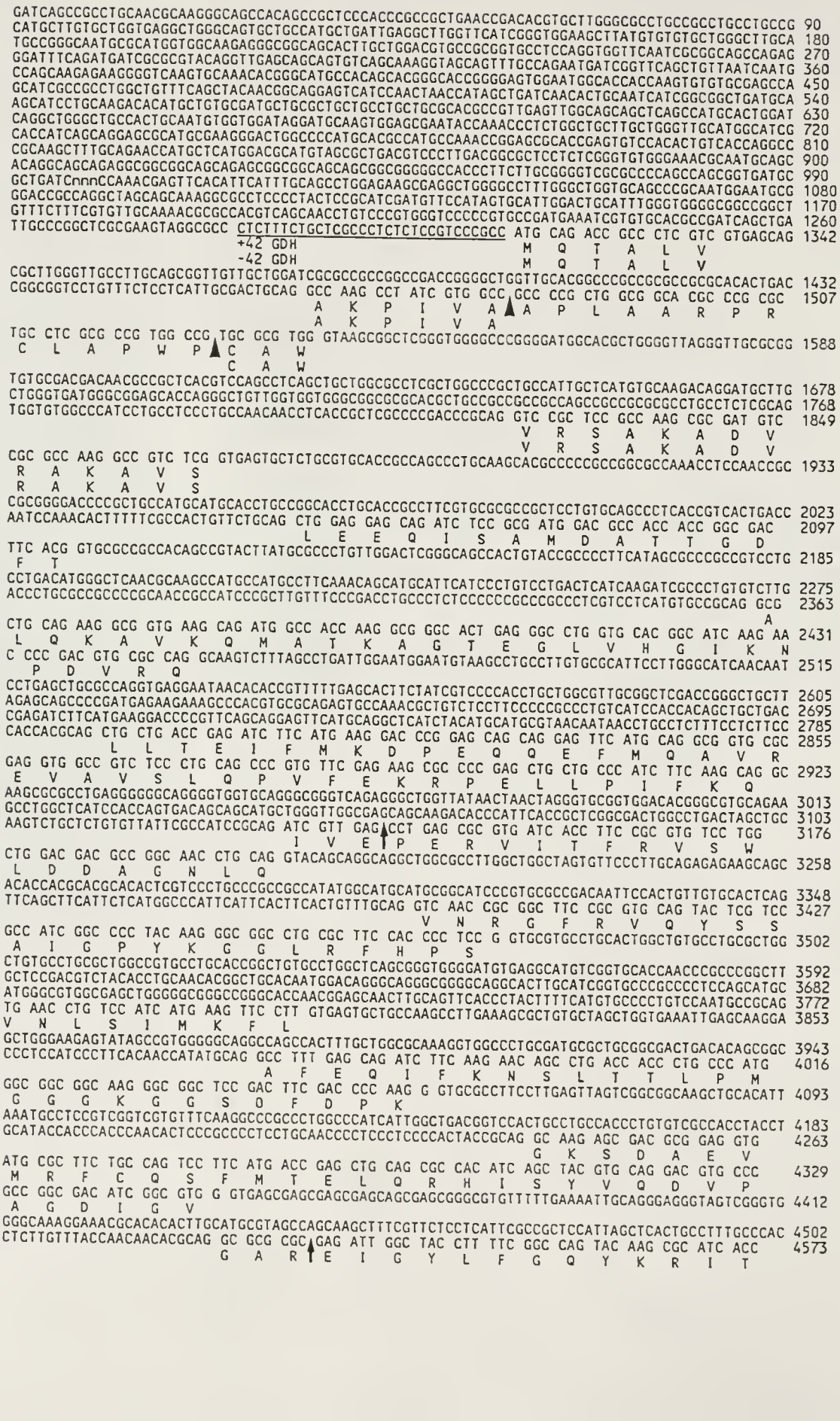

NH2
50
100
150
200
250
300
350
400
450
500
COOH
α-helix
β-sheet
Turn

Figure 9. Nucleotide sequence of the *C. sorokiniana* NADP-GDH gene containing 22 exons (+42 bp mRNA) or 23 exons (-42 bp mRNA). The position of the exons are identified by their corresponding deduced amino acid sequences. The 5'-VR amino acid sequences are indicated separately in the 5' region of the gene. The highly conserved glutamate binding region (Mattaj et al., 1982) is distributed over six exons encompassing 2.09 kb as indicated by the two arrows. The 5'-VR 42 bp auxon is denoted by arrowheads. Underlined regions indicate the 5'-UTR and 3'-UTR, respectively.



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TTGGGTGAACTGGTGTTCTCCAGCAGCCAGAGTTTTCCATGTCCACCCGCCTGCAAGCTCCTGGCTGTTCATCGCTGTGCTCTGTGTCTC 4832

CCTGCCAACACAATCCATACCAACACAATCCTGCGCCCTGCAG C CAG GAG TAT GGC GGC TCC GAG ATC CGC CCC GAG  4909
                                              Q  E  Y  G  G  S  E  I  R  P  E
GCC ACC GGC TAC GGC GCC GTG CTG TTT GTG GAG AAC GTG CTG AAG GAC AAG GGC GAG AGC CTC AAG GT 4977
 A  T  G  Y  G  A  V  L  F  V  E  N  V  L  K  D  K  G  E  S  L  K
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ATGAGGTAGAGCACAGCCCAAACTGCTCAGAACGTCCGCCTGCCAAGTTCTTTCTTCCATCCACACCCCACACACCTGTGCAG GGC    5153
                                                                                        G
AAG CGC TGC CTG GTG TCT GGC GCG GGC AAC GTG GCC CAG TAC TGC GCG GAG CTG CTG CTG GAG AAG      5219
 K  R  C  L  V  S  G  A  G  N  V  A  Q  Y  C  A  E  L  L  L  E  K
GGC GCC ATC GTG CTG TCG CTG TCC GAC TCC CAG GGC TAC GTG TAC GAG GTGCGGTTGATACATCTGGGCCATTT 5293
 G  A  I  V  L  S  L  S  D  S  Q  G  Y  V  Y  E
CGGCTGTTGATTGTGCTCTGTGTTGTCTGTAGTGTCTGACTCCCTGGGCTCGTGCACGAGGTGCGGAAGGCTCAGGCAGCAGTTCGGAGC 5383
TCTGCCTGTCTGCTGCTCCTAGAGCTACCTAATGAAGCATAGCTCTGCTGTGCTGCCCCCTCGCGCCTGCTCACCCGTCAACCACCACCG 5473
CCCCTCCCCACCCCCTTTTCATTTTTCCCGCAG CCC AAC GGC TTC ACG CGC GAG CAG CTG CAG GCG GTG CAG GAC  5548
                                 P  N  G  F  T  R  E  Q  L  Q  A  V  Q  D
ATG AAG AAG AAG AAC AAC AGC GCC CGC ATC TCC GAG TAC AAG GACAGTGATGACCGGTCCAGGAAACAAGTTGCAC 5624
 M  K  K  K  N  N  S  A  R  I  S  E  Y  K
ATGTCGTCTAGAAGGTCCCTGCCGCCGACACAGCAGCCGCGCCTGGGCTGCCGCTGCTTCGATAGCACCACCCACCCCTGCCGCCCCATC 5714
TCCTGCCTGCACTGCACCTTCCCATTTGCCCACTAGCCACTGCTCACTCGAGTTCTCAACTGTCACTTGCAATTTTCTCTCTGCTTGCA 5804
G AGC GAC ACC GCC GTG TAT GTG GGC GAC CGC CGC AAG CCT TGG GAG CTG GAC TGC CAG GTG GAC ATC    5871
   S  D  T  A  V  Y  V  G  D  R  R  K  P  W  E  L  D  C  Q  V  D  I
GCC TTC CCC TGC GCC ACC CAG GTGCGCAGCCAGACTGGCTTGCATGCAACGCATCAAATGTCTCAAGGTTTGCCTGCAAGTGC 5954
 A  F  P  C  A  T  Q
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                         N  E  I  D  E  H  D  A  E  L  L  I  K  H  G  C
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 Q  Y  V  V  E  G  A  N  M  P  S  T  N  E  A  I  H  K  Y  N  K
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CCCACCCTTCCCTCTCCCTGCCTCCGCGTCGCGCAG GCC GGC ATC ATC TAC TGC CCC GGC AAG GCG GCC AAC GCC    6620
                                     A  G  I  I  Y  C  P  G  K  A  A  N  A
GGC GGC GTG GCG GTC AGC GGC CTG GAG ATG ACC CAG AAC CGC ATG GTGAGCGTGGCATGATTTCCCTGCTTGTCA 6695
 G  G  V  A  V  S  G  L  E  M  T  Q  N  R  M
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GTTAACTGGGAGCAGCCTCAGCGACGCCCAGTGCTGGTGCTTTGTTCCTCGTGCACCTCAGCTGCTGCAACTTTGTGAGCGCATCGCCCT 6875
GAACCGCCACAACTGCCTGCGCCTGCCCTGCCGCAG AGC CTG AAC TGG ACT CGC GAG GAG GTT CGC GAC AAG CTG    6950
                                     S  L  N  W  T  R  E  E  V  R  D  K  L
GAG CGC ATC ATG AAG GTGAGGGCTGATTGTGCGGCTATCACAGTGCAACCACGCAAGCTGGAGCGCATCATGAAGGTGAGGGCTG 7035
 E  R  I  M  K
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GCAGGACAACCCATTCCGTTCACAACACTCAGCAACCCTGCCCTCATTCTTCTTCATCCCCGCAG GAC ATC TAC GAC TCC GCC  7298
                                                                    D  I  Y  D  S  A
ATG GGG CCG TCC CGC GAG TAC AAT GTT GAC CTG GCT G GTGAGTGCCTGGCTGTGCAGACAGACACGACACTTGTAAA 7375
 M  G  P  S  R  E  Y  N  V  D  L  A
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                                A  G  A  N  I  A
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     G  F  T  K  V  A  D  A  V  K  A  Q  G  A  V  *
GCTCACCGGCAATCCAACCCAACCAACTCAACGGCCAGGACCTTTTCGGAAGCGGCGCCTTTTTCCCAGCCAGGGCCCTCACCTGCCCTT 8070
TCATAACCCTGCTATTGCCGCCGTGCCCCTGCAATTCCACCCCAAGAAGAACTAGCGGCACTTGACTGCATCAGGACGGCTATTTTTTTC 8160
GCGACGCGCGCTCACCCCGAGAGCCTCTCTCCCCCGAGCCCTAAGCGCTGACGTCCGCCCGACTTTGCCTCGCACATCGCTCGGTTTTGA 8250
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GCATTTACAGTTCCAGACCCAGTCAGATCACCCAGGGCATCCACCAGCCACTGCAAAGCGGTTGCACAGCGGCTCGGCTCGATGGCGCCG 8699
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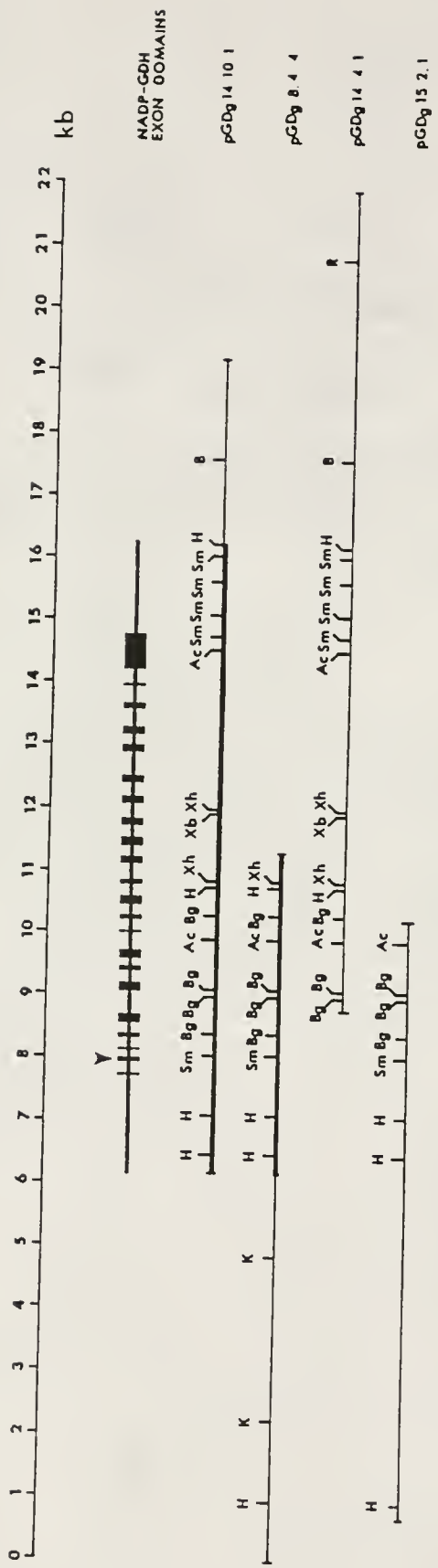
ACAAAGAAATCCCGGGGATCGTGGGCCGGTGAGCTGGGCGGTGGGGCATTCGCATGAACATGCATATCCTGGCTGCCACAGCGTGCCGCA 9779
TGCTATGTTGGGTGGGTCCACGGCAGTTGGCCGCTCGGTGCCGCTGGTGCATGCTGTGTGGGAGGGGAAGCTGCCTCGGTGTGACCTGAA 9869
GGACTT                                                                                    9873
```

Figure 9 continued

the *C. sorokiniana* genome and are divided into 22 exons and 23 exons, respectively (Fig. 10). The exons in this gene exhibit a range from 9 bp (exon 3 of the -42 nt mRNA) to a large exon of 550 bp at the 3' end of the gene. The 9, 16, and 18 bp exons are smaller than the smallest exons identified in higher-plant genes (Tischer et al., 1986).

The introns identified in the NADP-GDH gene range between 42 and 402 bp with a mean length of 233 bp. The mean intron length is similar to the mean intron length of 249 bp calculated in a survey of higher-plant introns (Hawkins, 1988). The consensus sequence $\begin{matrix} C & & A & TC \\ AG/GTG & & GG & \\ A & & C & CG \end{matrix}$, was derived as the 5' intron donor site using the criteria suggested by Cavener (1987). The 5' donor site closely matches the general consensus, CAG/GTAAGT observed in higher-plants and animals (Hawkins, 1988) with the exception of G at position +3 of the *C. sorokiniana* intron. Three of the 21 conserved 5' intron splice junctions do not conform to the GT-AG rule of Breathnach and Chambon (1981) due to a substitution of C at position +2. A consensus sequence, $\begin{matrix} & & TTTTTTT & & T\ T \\ C & & CcCtt & C & GCAG/, \\ & & CCCCCCC & & C\ C \end{matrix}$ was derived for the 3' intron splice junction of the NADP-GDH gene. The strong preference for G at position -4 observed in 19 of 21 conserved intron splice junctions is typical of plant introns (Brown, 1986). The 5' (GCC/GCC) and 3' (CCG/TGC) splice site junctions of the 42 bp auxillary exon (termed auxon hereafter as per Werneke et al. [1989]) showed no conservation in splice site branch points.

Figure 10. Restriction maps and exon domains of four NADP-GDH genomic clones spanning 21.9 kbp of the *C.sorokiniana* genome. The exons (black boxes) are interupted by introns with consensus exon/intron junction splice sites with the exception of the alternatively-spliced region denoted in exon two (arrow). Heavy black lines denote regions in genomic clones that have been sequenced on both strands. Restriction maps were generated using *Acc*I (Ac), *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (R), *Hind*III (H), *Kpn*I (K), *Sma*I (Sm), *Xba*I (Xb), and *Xho*I (Xh). Primary genomic cloning and NADP-GDH gene sequencing performed by Drs. Mark Cock and Kyu Don Kim with revisions provided by the author (Cock et al., 1991).


kb
NADP-GDH EXON DOMAINS
pGDg 14 10 1
pGDg 8.4 4
pGDg 14 4 1
pGDg 15 2.1

Analysis of the 1285 bp sequence upstream from the transcriptional start site revealed no consensus cis-regulatory elements common with those published for other eukaryotic organisms (Fig. 11).

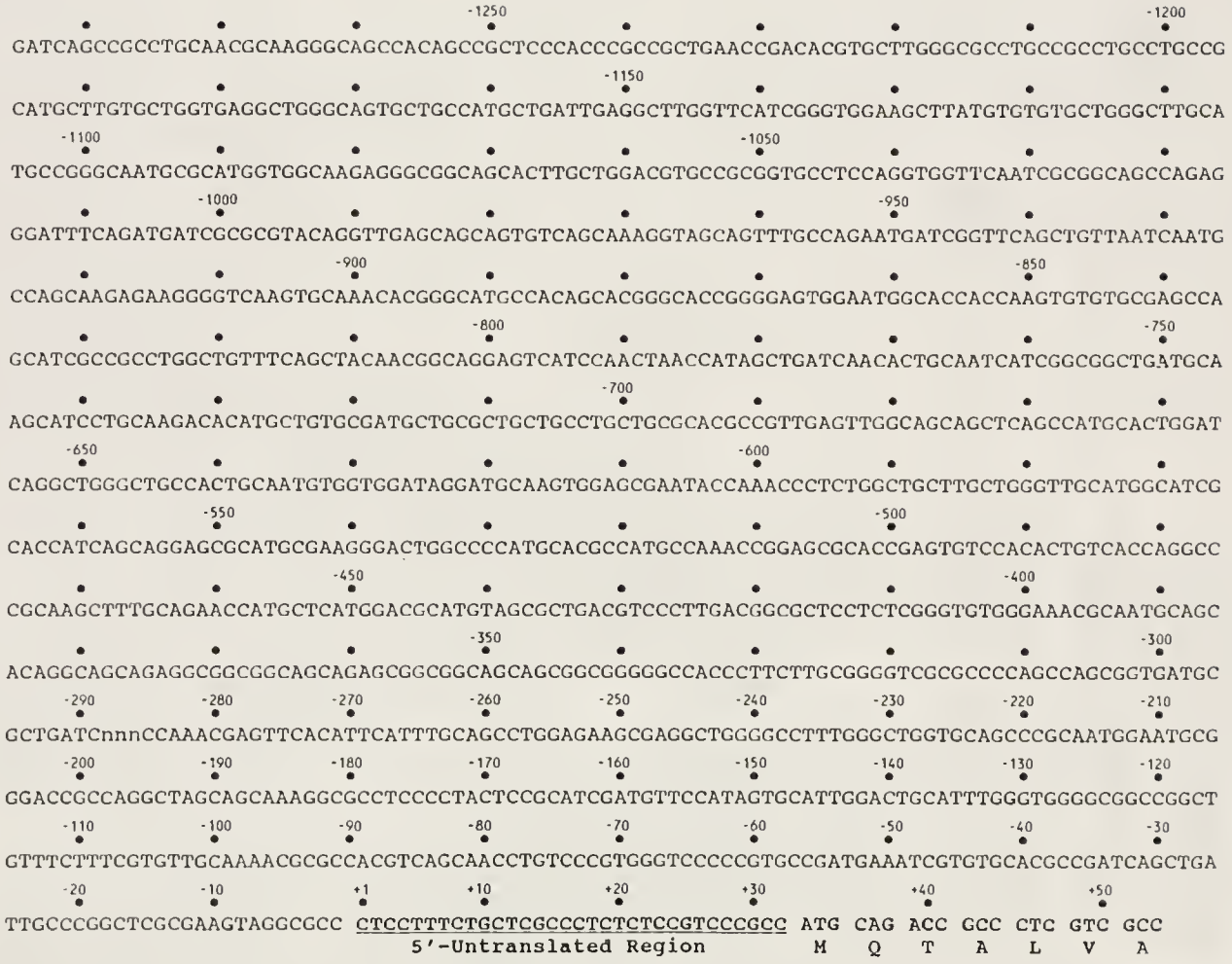
Determination of the Number of NADP-GDH Genes in the *C. sorokiniana* Genome

Southern blot analysis of the NADP-GDH gene

Using cDNA probes corresponding to the HCR and 3'-UTR, Cock et al. (1991) provided evidence that indicated the *C. sorokiniana* genome possessed a single NADP-GDH gene. However, the possiblity remained that two recently diverged GDH genes differing only in their 5' ends might exist in the *C. sorokiniana* genome. *C. sorokiniana* genomic DNA was digested with *Pvu*II or *Taq*I and the resulting restriction fragments were subjected to Southern blot analysis using cDNA probes that hybridized to the 5'-VR (exons 1 to 3), HCR (exons 10 and 11), and the 3'-UTR (exon 22) of the NADP-GDH gene. The respective restriction enzyme and predicted hybridizing fragment sizes were as follows: 5'-VR *Pvu*II 634, 429, 367 bp *Taq*I 1465 bp; HCR *Pvu*II 2646 bp *Taq*I 590 bp, 1159 bp; 3'-UTR *Pvu*II 4300 bp *Taq*I 1334 bp.

The 5'-VR and 3'-UTR probes yielded a hybridization pattern that was predicted from the sequenced NADP-GDH gene (Fig. 12). The HCR probe gave the predicted hybridization pattern; however, an additional weakly hybridizing fragment

Figure 11. Transcriptional initiation site for the *C. sorokiniana* NADP-GDH nuclear gene and the upstream region in the genomic DNA that contains the putative NADP-GDH promoter. Also shown is the 5'-UTR and the first seven amino acid codons identified in the two NADP-GDH mRNAs. The entire region of genomic DNA was derived from genomic clone 14.10.1 (Fig. 10). The identification of the transcription initiation site, 5'-UTR, start codon, and other amino acid codons was acheived by computer alignment with the 5' RACE-PCR generated cDNA fragments with the NADP-GDH gene sequence. In the 1285 sequence upstream of the transcriptional start site, no strong consensus cis-regulatory elements common with those published for other eukaryotic organisms were identified (Miller et al., 1994b).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-1200
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-850
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CAGGCTGGGCTGCCACTGCAATGTGGTGGATAGGATGCAAGTGGAGCGAATACCAAACCCTCTGGCTGCTTGCTGGGTTGCATGGCATCG
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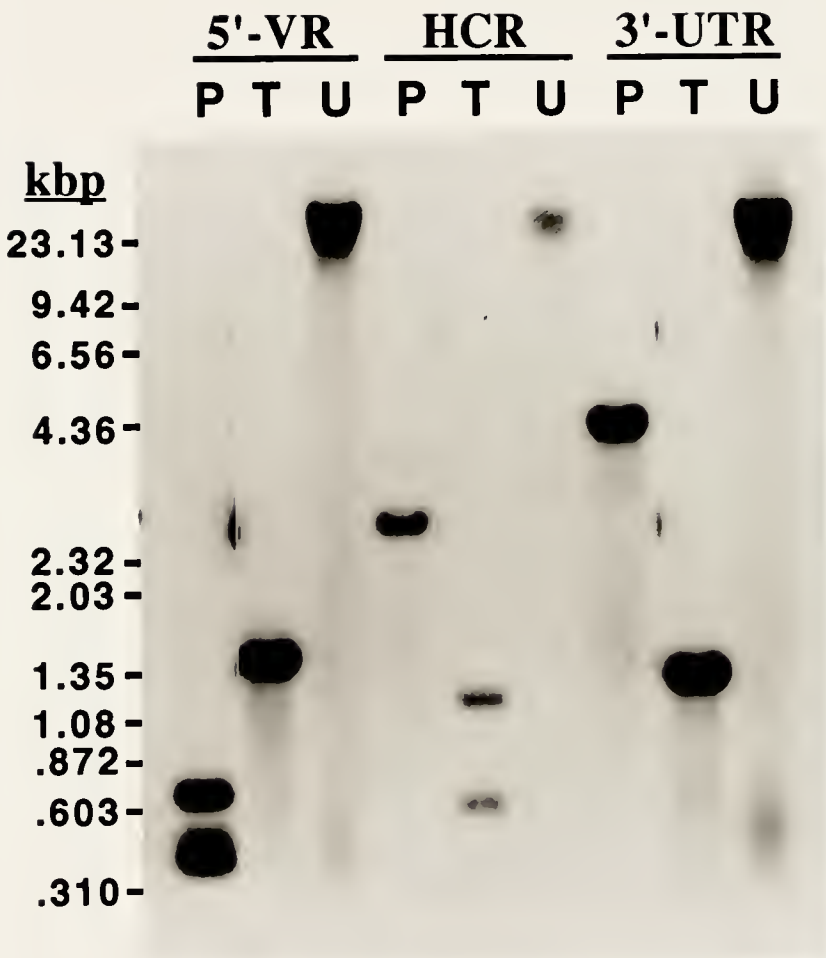
-500
CACCATCAGCAGGAGCGCATGCGAAGGGACTGGCCCCATGCACGCCATGCCAAACCGGAGCGCACCGAGTGTCCACACTGTCACCAGGCC
-450
-400
CGCAAGCTTTGCAGAACCATGCTCATGGACGCATGTAGCGCTGACGTCCCTTGACGGCGCTCCTCTCGGGTGTGGGAAACGCAATGCAGC
-350
-300
ACAGGCAGCAGAGGCGGCGGCAGCAGAGCGGCGGCAGCAGCGGCGGGGGCCACCCTTCTTGCGGGGTCGCGCCCCAGCCAGCGGTGATGC
-290
-280
-270
-260
-250
-240
-230
-220
-210
GCTGATCnnnCCAAACGAGTTCACATTCATTTGCAGCCTGGAGAAGCGAGGCTGGGGCCTTTGGGCTGGTGCAGCCCGCAATGGAATGCG
-200
-190
-180
-170
-160
-150
-140
-130
-120
GGACCGCCAGGCTAGCAGCAAAGGCGCCTCCCCTACTCCGCATCGATGTTCCATAGTGCATTGGACTGCATTTGGGTGGGGCGGCCGGCT
-110
-100
-90
-80
-70
-60
-50
-40
-30
GTTTCTTTCGTGTTGCAAAACGCGCCACGTCAGCAACCTGTCCCGTGGGTCCCCCGTGCCGATGAAATCGTGTGCACGCCGATCAGCTGA
-20
-10
+1
+10
+20
+30
+40
+50
TTGCCCGGCTCGCGAAGTAGGCGCC CTCCTTTCTGCTCGCCCTCTCTCCGTCCCGCC ATG CAG ACC GCC CTC GTC GCC
5'-Untranslated Region M Q T A L V A

Figure 12. Southern blot analysis of undigested genomic DNA (U) and restriction fragments obtained by digestion of *C. sorokiniana* genomic DNA with *Pvu*II (P), and *Taq*I (T). The blots were hybridized under stringent conditions to cDNA probes derived from the 5'-VR, HCR, and the 3'-UTR of the NADP-GDH cDNAs. The stringency of the washes was 0.1x SSC, 0.1% SDS at 65°C.


5'-VR
HCR
3'-UTR
P T U P T U P T U
kbp
23.13
9.42
6.56
4.36
2.32
2.03
1.35
1.08
.872
.603
.310

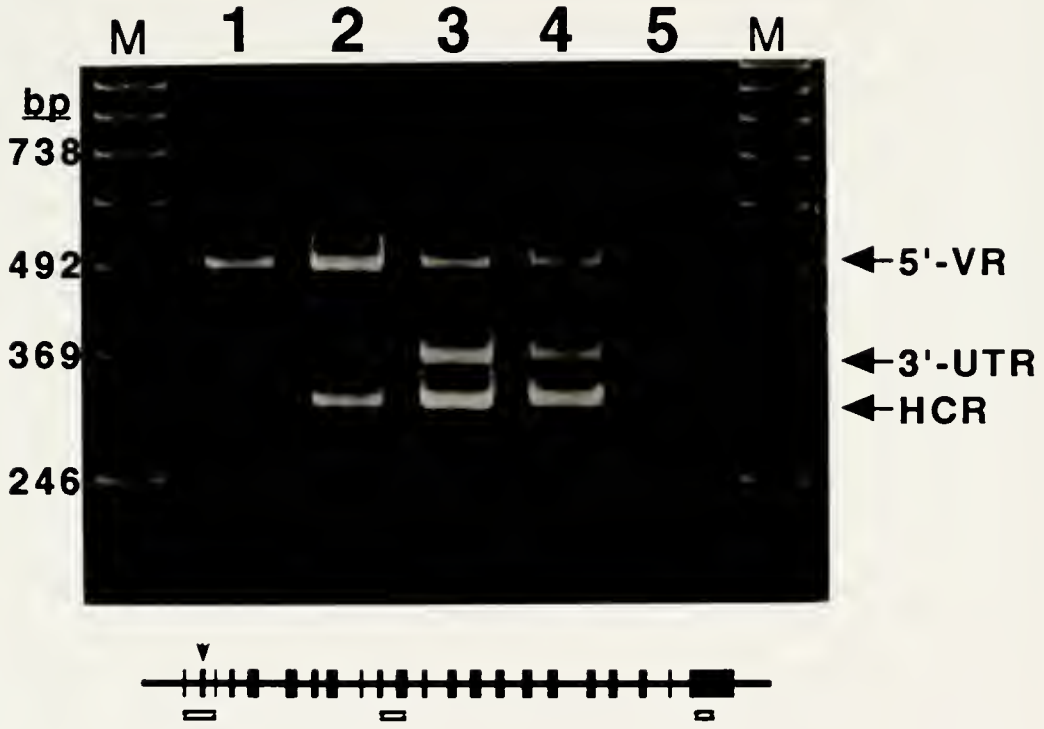
was detected in both the *Pvu*II and *Taq*I digest that could not be explained by incomplete restriction digestion (Fig. 12). Since the 155 bp NADP-GDH HCR probe is derived from the highest region of conservation in the glutamate binding domain, the faintly hybridizing band most likely corresponds to the *C. sorokiniana* mitochondrial NAD-GDH gene (Meredith and Schmidt, 1991). These results provide evidence for the existence of a single NADP-GDH gene in the *C. sorokiniana* genome.

Allele-specific PCR analysis of the NADP-GDH gene

Since the Southern blot analysis is limited by the resolution capability of agarose gel electrophoresis, the remote possibility remained that two NADP-GDH genes exist that only differ in their 5' ends by the 42 bp detected in the NADP-GDH +42 nt mRNA. In order to test this possibility, *C. sorokiniana* genomic DNA was analyzed by allele-specific PCR (Saiki et al., 1986). Genomic DNA and three NADP-GDH genomic clones spanning various regions of the NADP-GDH gene (pGDg 8.4.4, 14.10.1, 15.2.2; Fig. 10) were amplified using a series of exon specific primers. The primers were designed to anneal to the 5'-VR (exons 1 and 3), HCR (exons 10 and 11), and 3'-UTR (exon 22) of the NADP-GDH gene. The resulting PCR products were resolved by high-resolution PAGE and analyzed for DNA polymorphisms (Fig. 13).

Allele-specific PCR of *C. sorokiniana* genomic DNA yielded a single PCR fragment corresponding to the 5'-VR (564

Figure 13. Polyacrylamide gel electrophoresis of the PCR products amplified from *C. sorokiniana* genomic DNA and three NADP-GDH genomic clones, lacking or possessing the regions of interest, using exon-specific primer pairs. Primer pairs hybridized to exons 1 and 3 (5'-VR), exons 10 and 11 (HCR), and the last 3' exon of the NADP-GDH gene (3'-UTR). Lanes: 1, pGDg 15.2.1; 2, pGDg 8.4.4; 3, pGDg 14.10.1; 4, *C. sorokiniana* genomic DNA; 5, pUC 18 control template. A single PCR fragment of the predicted size was amplified from the genomic DNA template with each set of exon specific primers. PCR products for a given template were combined before separation by PAGE. Arrow denotes (lower figure) location of the 42 nt auxon.


M
1
2
3
4
5
M
bp
738
492
369
246
5'-VR
3'-UTR
HCR

bp), HCR (344 bp), and 3'-UTR (387 bp). The products generated from the genomic clones template DNA yielded identical products to the total genomic DNA (Fig. 13) and were identical in size to the sizes predicted from the NADP-GDH gene sequence. These results provide strong evidence that the two NADP-GDH mRNAs are derived from a single NADP-GDH gene rather than two closely related genes.

Purification of the NADP-GDH Isoenzymes

The NADP-GDH α-holoenzyme was purified from a 130 g pellet of *C. sorokiniana* cells induced continuously in 29 mM ammonium medium according to Yeung et al. (1981). The final step of purification was modified from that of Yeung et al. (1981) by substituting a nondenaturing preparative PAGE step (Miller et al., 1994) for a NADP-hexane-agarose affinity chromatography step. This procedural alteration was required due to the lack of availability of the affinty agarose. Approximately 11.5 mg of pure NADP-GDH α-holoenzyme with a specific activity of 254 units/mg protein was obtained from the 130 g of cells with only a 17% loss of the initial activity (Table 4). The nondenaturing PAGE step effectively purified the the enzyme an additional 8.5-fold over the Sephadex G-200 step with less than a 2% loss of activity. A single protein band of 53.5 kD corresponding to the NADP-GDH α-subunit was detected by silver-staining after resolution by Tris-Tricine SDS-PAGE (Fig. 14).

Table 4. Steps for the purification of the NADP-GDH α-holoenzyme

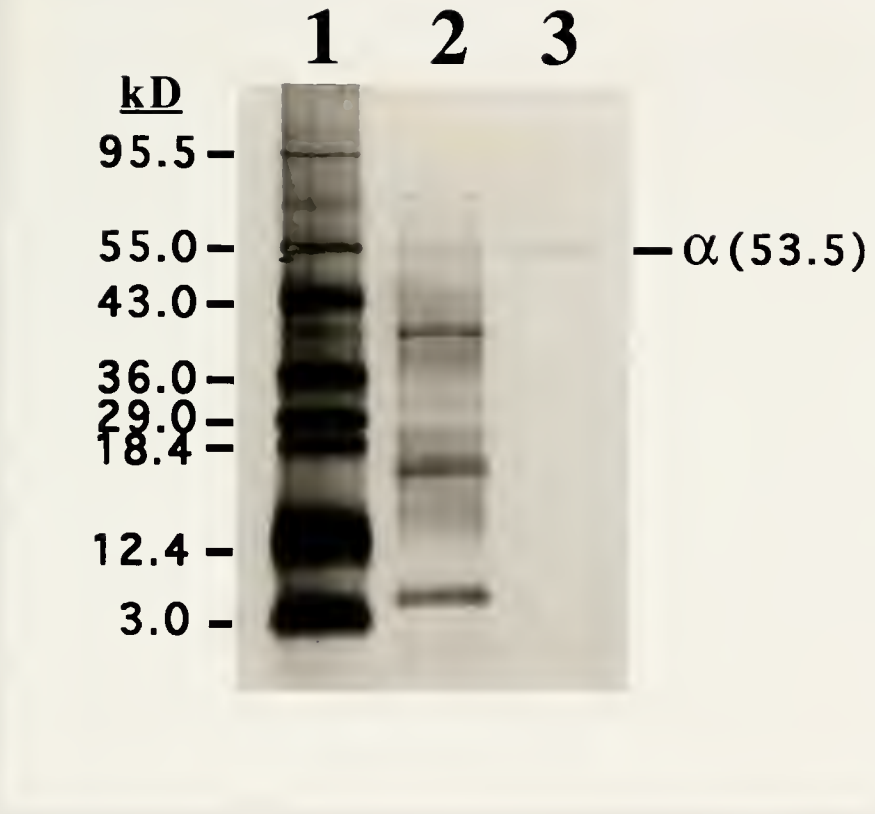
Step	Total Units[a]	Sp. Activity[b]	% Recovery	Fold Purification[c]
Supernatant frozen/thawed cell homogenate	3546	0.678	100	0
Ammonium sulfate fractionation (35%- 70%)	3526	1.22	99	1.8
DEAE-Sephacel pH 7.4	3411	5.16	96	7.6
DEAE-Sephacel pH 6.0	3060	23	86	33.9
Sephadex G-200	3011	30	85	44.2
Model 491 preparative native PAGE	2938	254	83	375

[a]One Unit is defined as the amount of enzyme necessary to reduce 1μmol of $NADP^+$/min at 38.5°C

[b]Specific activity is defined as units/mg protein

[c]Fold purification is defined as the specific activity of step/specific activity of supernatant cell homogenate

Figure 14. Analytical SDS-PAGE of the NADP-GDH α-holoenzyme purified by preparative nondenaturing gel electrophoresis. Proteins were detected by rapid silver-staining. Lanes: 1, Molecular mass standards; 2, Sephadex G-200 NADP-GDH fraction; 3, Nondenaturing preparative gel purified NADP-GDH α-subunit (53.5 kD).


1
2
3
kD
95.5
55.0
43.0
36.0
29.0
18.4
12.4
3.0
α(53.5)

A mixture of NADP-GDH isoenzymes containing both the α- and β-subunit were functionally purified from a 24 g pellet of a mixture of *C. sorokiniana* cells cultured under various time regimes and ammonium concentrations. The NADP-GDH isoenzymes were purified using a modified procedure of Yeung et al. (1981). In this procedure, all column volumes were decreased ten-fold and the Sephadex G-200 column was omitted (Table 5).

The isoenzyme preparation was purified 116-fold to a specific activity of 45 units/mg protein with a loss of 37.5% of the initial activity. The greater loss of activity observed in the isoenzyme mixture during the purification procedure may be in part due to the presence of a more labile heterohexameric and/or β-homohexameric form. Silver-stained Tris-Tricine SDS gels of the partially purified enzyme preparation revealed that the preparation still contained several contaminating proteins. However, the NADP-GDH α- and β-subunits were sufficiently resolved from the contaminants to allow further protein analyses.

Determination of the Stability of the NADP-GDH α-Holoenzyme in the Presence of $NADP^+$

Purified NADP-GDH was reported to undergo polymerization and inactivation during storage at -20°C (Yeung et al., 1981). Since many studies require the longterm use of catalytically active NADP-GDH, a means of storage without enzyme activity loss was developed. Several researchers in this laboratory observed a stabilization of NADP-GDH activity

Table 5. Steps for the partial purification of NADP-GDH isoenzymes

Step	Total Units[a]	Sp. Activity[b]	% Recovery	Fold Purification[c]
Supernatant frozen/thawed cell homogenate	320	0.387	100	0
Ammonium sulfate fractionation (35%- 70%)	310	0.812	97	2.1
DEAE-Sephacel pH 7.4	272	2.67	88	6.9
DEAE-Sephacel pH 6.0	183 71	9.28	79	24
NADP+ Type 3 Affinity Resin	132 68	45	62.5	116

[a]One Unit is defined as the amount of enzyme necessary to reduce 1μmol of $NADP^+$/min at 38.5°C

[b]Specific activity is defined as units/mg protein

[c]Fold purification is defined as the specific activity of step/specific activity of supernatant cell homogenate

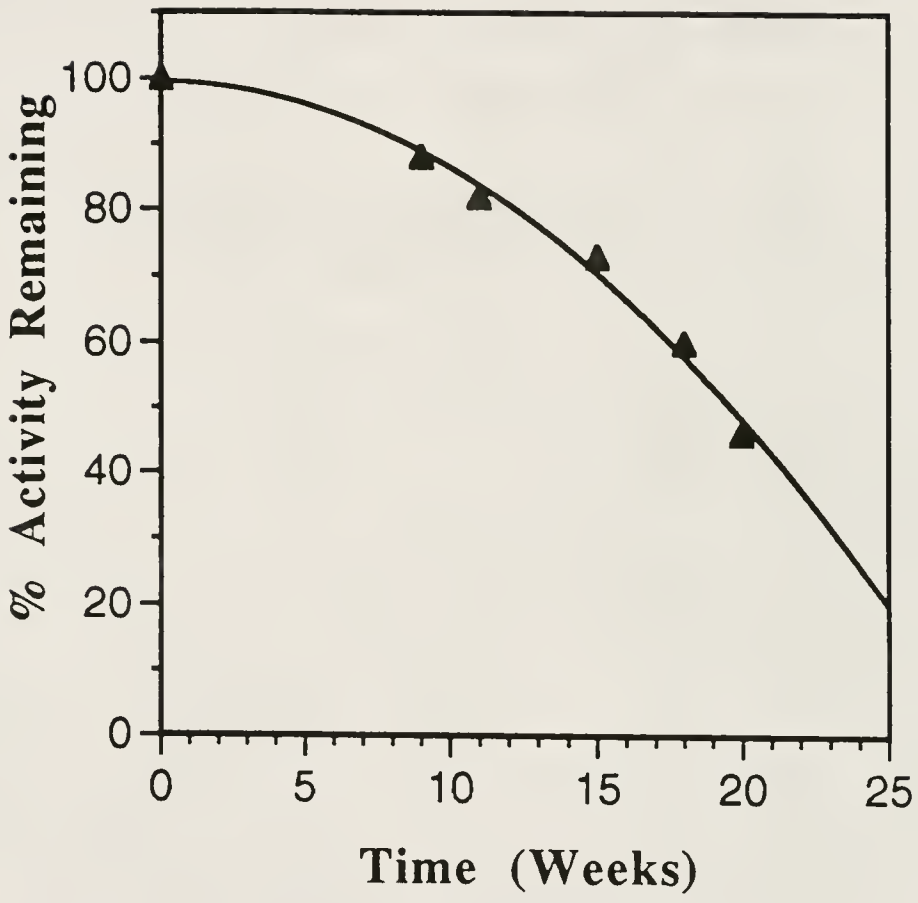
in the presence of one of the cofactors, $NADP^+$. To test the ability of NADP+ to stabilize the GDH enzyme activity during storage at 4°C, 205 μg (52 units) of purified α-holoenzyme in 10 mL of 10 mM KPO4 (pH 6.2), 2 mM DTT, and 0.1 mM $NADP^+$ was placed at 4°C and periodically tested for NADP-GDH deaminating activity. The purified NADP-GDH retained 90% of the original activity after 10 weeks of storage and 50% of the activity was lost after 20 weeks storage (Fig. 15). Thus, the NADP-GDH α-holoenzyme can be stored in the presence of NADP+ at 4°C for extended periods of time without a rapid loss of enzyme activity.

Production of Anti-NADP-GDH Polyclonal and Monoclonal Antibodies

Polyclonal rabbit anti-NADP-GDH antibodies were produced against the α-holoenzyme in six rabbits. Preimmune sera and production bleed anti-sera were titered by their ability to immunoprecipitate one unit of NADP-GDH at 22°C. All six anti-sera possessed the ability to immunoprecipitate the NADP-GDH activity to varying degrees in a dilution range from 1×10^{-4} to 2%. The preimmune sera lacked the ability to precipitate the GDH activity and was shown to stabilize the GDH activity at 22°C when compared to a control that lacked serum. The anti-serum possessing the highest titer was selected for use in western blot analyses.

Monoclonal antibodies were produced from splenocytes of female Balb/C mice immunized with the NADP-GDH α-subunit

Figure 15. Stability of purified NADP-GDH α-holoenzyme at 4°C in the presence of 0.1 mM $NADP^+$. A sample of purified α-holoenzyme was stored at 4°C in 10 mM KPO4, 2 mM DTT, and 0.1 mM $NADP^+$ and periodically analyzed to determine the amount of NADP-GDH deaminating activity that remained. NADP-GDH deaminating activity was measured at 38.5°C by a spectrophotometric analysis.


% Activity Remaining
100
80
60
40
20
0
0
5
10
15
20
25
Time (Weeks)

(Tamplin et al., 1991). Splenocyte-myeloma fusions produced more than 1000 hybridomas. A total of 12 hybridomas initially tested ELISA positive for producing anti-NADP-GDH antibodies; however, only nine of the hybridoma lines were stable. Results of western blot tests of hybridoma supernatants showed that all nine hybridomas were producing antibodies capable of detecting the denatured α-subunit immobilized on membranes. Hybridoma supernatants were collected and stored at -20°C.

Analysis of the α- and β-subunit similarity with Mouse Anti-NADP-GDH MAbs

Yeung et al. (1981) demonstrated that rabbit polyclonal antibodies derived against the NADP-GDH β-holoenzyme were able to immunoprecipitate both the α- and β-subunits of the NADP-GDH. Futhermore, Bascomb and Schmidt (1987) revealed by peptide mapping that the two subunits had 36 of 40 peptides in common. To test further the hypothesis that the α- and β-subunits are nearly identical, a protein preparation possessing both subunit types was analyzed for cross-reactivity with MAbs derived against the α-subunit. Proteins in a partially purified mixture of NADP-GDH isoenzymes were resolved by Tris-Tricine SDS PAGE and transferred to a nylon membrane. The blot was cut into strips and each strip was incubated individually with one of five mouse anti-NADP-GDH MAbs. All five (MAb: GDH 1, 3, 4,8, 9) of the anti-NADP-GDH α-subunit MAbs detected both the α- and β- subunit on western

blots (Fig. 16; MAb GDH 4,8 not shown). These results provide further support for the high degree of similarity between the two subunit types.

Determination of the Molecular Mass of the NADP-GDH Subunits

Prunkard et al. (1986) determined the molecular mass of the NADP-GDH subunits, based on their mobilities in Tris-glycine SDS-PAGE, to be 55.5 kD and 53 kD for the α-subunit and β-subunit, respectively. In this study, it was determined that the earlier molecular mass estimations were in error. To determine the molecular masses of the NADP-GDH subunits, an aliquot of a partially purified protein preparation was resolved by Tris-Tricine SDS-PAGE and the position of each subunit was determined by immunodetection with mouse anti-NADP-GDH antibodies. The mobilities of the subunits were compared to the moblilities of molecular mass standards ranging from 69 to 30 kD. The relative mobilities of the standards were plotted against the molecular mass and the slope of the line was statistically computed (Fig. 17). The α-subunit and β-subunit sizes were calculated from the slope to be 53.48 kD and 52.27 kD, respectively. These values are in close agreement with the molecular mass predicted for the subunits from the deduced amino acid sequences (53.5 and 52.3 kD) of the mature subunits discussed later.

Figure 16. Mouse anti-NADP-GDH monoclonal antibody immunoblot analysis of the NADP-GDH α- and β-subunit similarity. MAbs developed against purified NADP-GDH α-subunit were tested for their ability to immunoreact with both the α- and β-subunits. Lanes: 1, MAb GDH 1; 2, MAb GDH 3; 3, MAb GDH 9. MAb:NADP-GDH subunit complexes were detected with alkaline-phosphate conjugated goat anti-mouse antibodies. All MAbs tested immunoreacted with both subunit types.

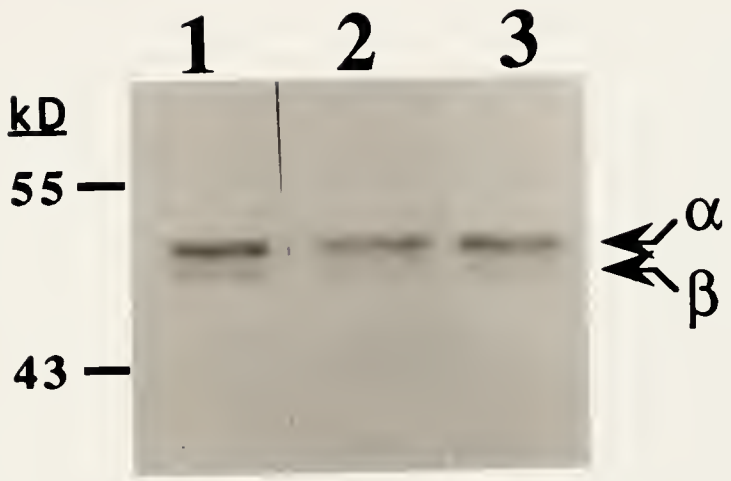
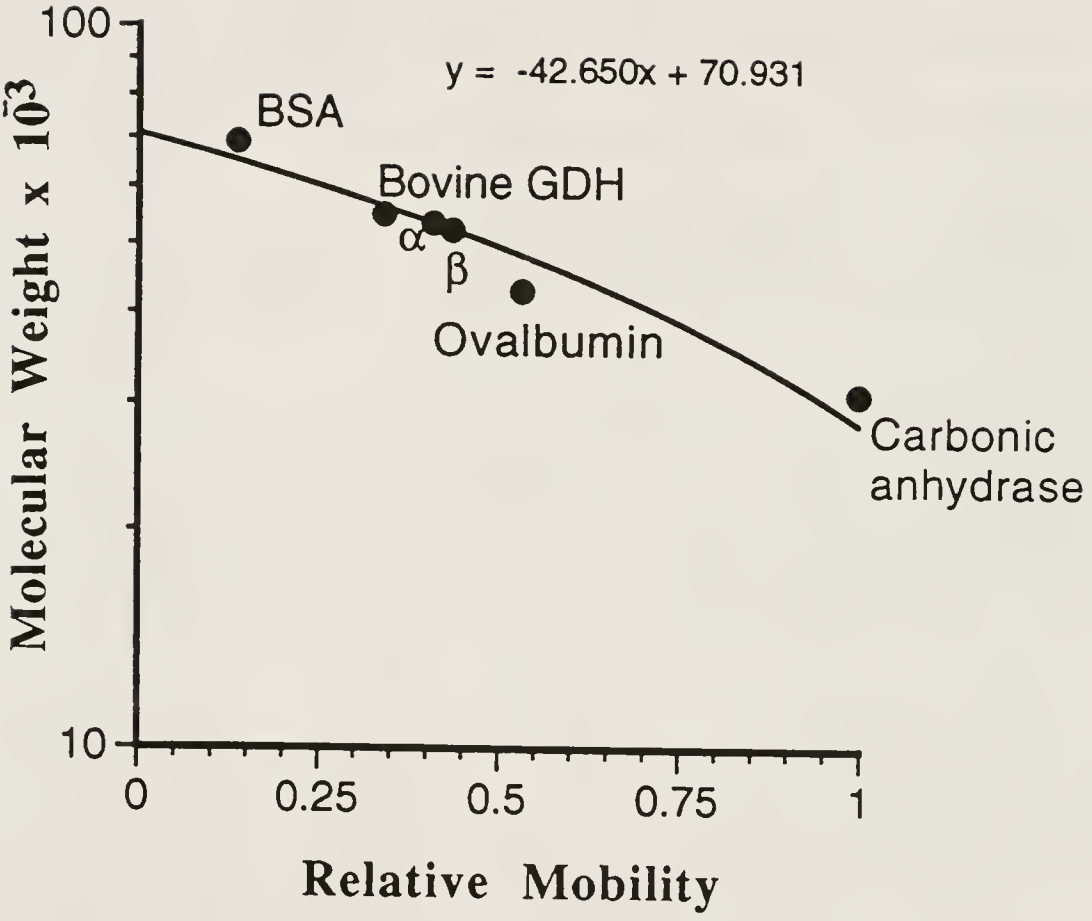

1
2
3
kD
55
43
α
β

Figure 17. Estimation of the molecular weights of the *C. sorokiniana* NADP-GDH α- and β-subunits. NADP-GDH subunits synthesized *in vivo* were partially purified and electrophoresed on a 8% SDS polyacrylamide gel and detected by immunoblotting. The molecular weight of the NADP-GDH α- and β-subunit was determined to be 53.48 and 52.27 kD, respectively.


100
10
y = -42.650x + 70.931
BSA
Bovine GDH
α
β
Ovalbumin
Carbonic
anhydrase
Molecular Weight x 10^3
0
0.25
0.5
0.75
1
Relative Mobility

Determination of the Amino-terminal Amino Acid Residues of the α- and β-Subunits

In order to identify the amino-terminal sequences of the mature NADP-GDH α- and β-subunits and to locate the transit peptide cleavage sites, the amino-terminus of both subunits was sequenced by automated Edman degradation. Purified α-subunit, and a second functionally pure mixture of α- and β-subunits were separated by Tris-Tricine SDS-PAGE and transferred to a PVDF membrane. The amino acid composition of the amino-terminal sequences were determined according to Plough et al. (1989).

A total of 20 amino-terminal amino acid residues of the α-subunit were identified: AVSLEEQISAMDATTGDFTA. Ten of the amino-terminal residues of the β-subunit were identified: DATTGDFTAL. In order to verify that the higher molecular weight band (53.5 kD) in the partially purified NADP-GDH mixture was in fact the same subunit sequenced from the purified α-subunit preparation, five amino-terminal residues were analyzed a second time. All five residues agreed with the aforementioned α-subunit amino-terminal residues.

Comparison of the α-subunit and β-subunit amino-terminal sequences revealed that the β-subunit lacked 11 amino-terminal amino acid residues identified in the α-subunit. Furthermore, comparison of the amino acid residues of the mature subunits to the deduced amino acid sequences of the NADP-GDH cDNAs predicted the α-subunit to be 53,501 D and the β-subunit to be 52,342 D. The 1159 D difference in the two

subunits was due to the additional amino-terminal residues in the α-subunit. The deduced molecular mass of the two subunits are extremely close to those predicted by SDS gel analysis.

The transit peptide SPP cleavage sites were identified:

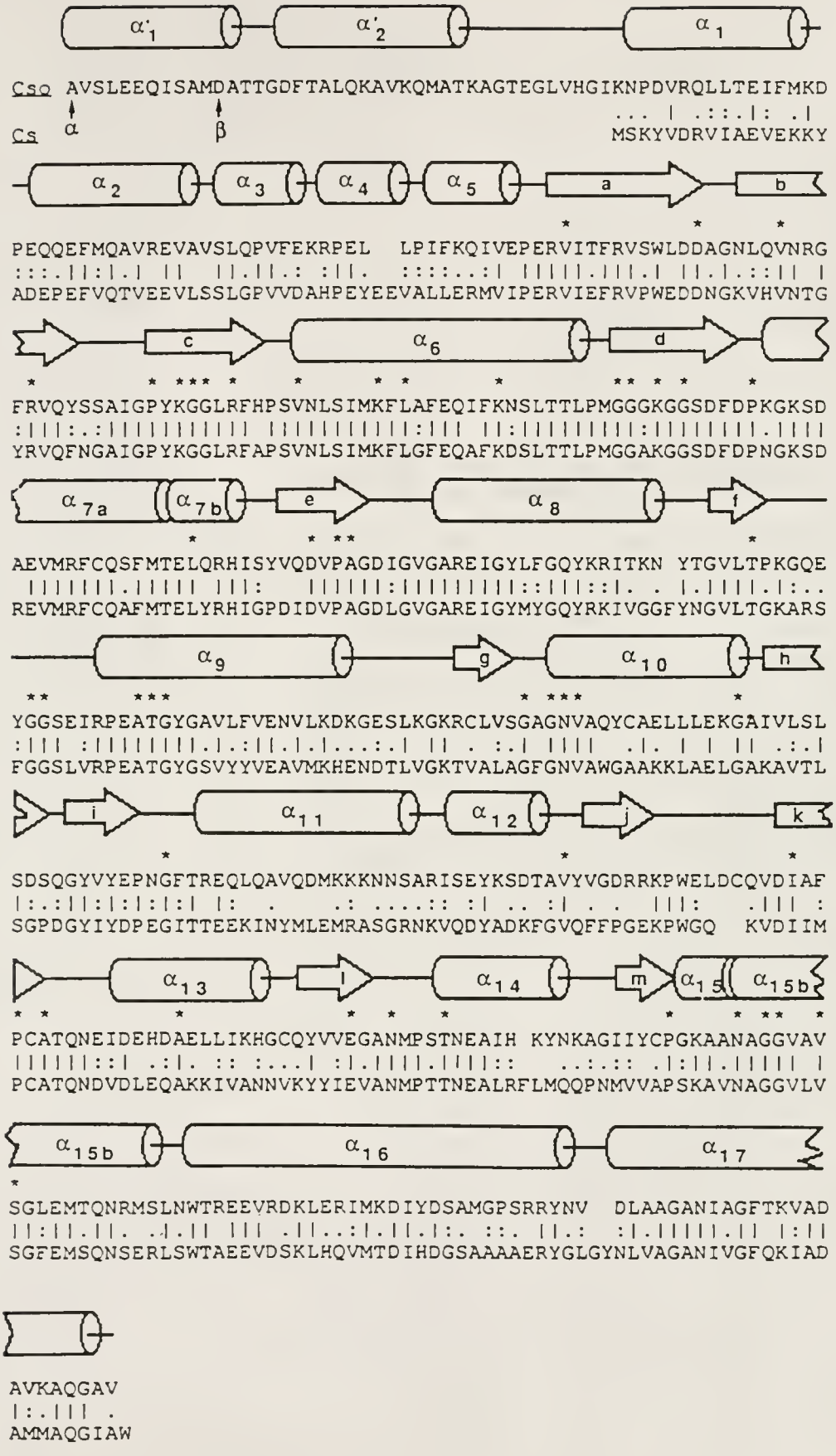
MQTALVAKPIVAAPLAARPRCLAPWPCAWVRSAKRDVRAK AVSLEEQISAM DATTGDFT
VR amino acids ^α ^β

Since both SPP cleavage sites were located downstream of the 14 amino acid variable region, it was not possible to determine by comparison if one or both of the two precursor proteins are processed to yield the α- or β-subunits, respectively.

All the protein data presented thus far support the strong physical similarities of the α- and β-subunits. In an attempt to explain the kinetic and biochemical differences observed between the α- and β-homohexameric isoenzymes (Bascomb and Schmidt, 1987), a comparison of the structural predictions of the two subunits was performed. Structural predictions were made according to Garnier et al. (1978) and by comparison to the published 1.96Å crystal structure of the *C. symbiosum* NAD-GDH (Baker et al., 1992).

The 486 amino acid residues comprising the α-subunit are organised into two domains. Domain one consists of residues 42 to 238 and residues 460 to 486 (Fig. 18). The second domain spans residues 239 to 406. The two helicies α_{15} and α_{16} (residues 407-459) serve as a link between the two domains. Domain one contains the glutamate-binding domain

Figure 18. Alignment of the *C. sorokiniana* NADP-GDH α- and β-subunit deduced amino acid sequences (Cso) with the *C. symbiosum* NAD-GDH amino acid sequence (Cs). The secondary structural elements of the NADP-GDH subunits are shown above the aligned sequences, with helicies represented as cylinders and strands shown as arrows. Amino acid residues are numbered in relation to the NADP-GDH α-subunit. The amino-terminal amino acid residues of the α- and β-subunits are denoted by small arrows and residues highlighted by asterisks (*) are conserved in the majority of GDHs sequenced to date. The GDH structural elements are labelled in reference to the three-dimensional structural elements identified in the *C. symbiosum* NAD-GDH (Britton et al., 1992). Structural elements α'_{1-2} (residues 1 to 41) are unique to the C. sorokiniana NADP-GDHs. Amino acid identities are denoted by vertical lines, high degrees of similarity are denoted by colons, and low degrees of similarity by periods.


α'1 α'2 α1
Cso AVSLEEQISAMDATTGDFTALQKAVKQMATKAGTEGLVHGIKNPDVRQLLTEIFMKD 57
α β
Cs MSKYVDRVIAEVEKKY
α2 α3 α4 α5 a b
PEQQEFMQAVREVAVSLQPVFEKRPEL LPIFKQIVEPERVITFRVSWLDDAGNLQVNRG 116
ADEPEFVQTVEEVLSSLGPVVDAHPEYEEVALLERMVIPERVIEFRVPWEDDNGKVHVNTG
c α6 d
FRVQYSSAIGPYKGGLRFHPSVNLSIMKFLAFEQIFKNSLTTLPMGGGKGGSDFDPKGKSD 177
YRVQFNGAIGPYKGGLRFAPSVNLSIMKFLGFEQAFKDSLTTLPMGGAKGGSDFDPNGKSD
α7a α7b e α8 f
AEVMRFCQSFMTELQRHISYVQDVPAGDIGVGAREIGYLFGQYKRITKN YTGVLTPKGQE 237
REVMRFCQAFMTELYRHIGPDIDVPAGDLGVGAREIGYMYGQYRKIVGGFYNGVLTGKARS
α9 g α10 h
YGGSEIRPEATGYGAVLFVENVLKDKGESLKGKRCLVSGAGNVAQYCAELLLEKGAIVLSL 298
FGGSLVRPEATGYGSVYYVEAVMKHENDTLVGKTVALAGFGNVAWGAAKKLAELGAKAVTL
i α11 α12 j k
SDSQGYVYEPNGFTREQLQAVQDMKKKNNSARISEYKSDTAVYVGDRRKPWELDCQVDIAF 359
SGPDGYIYDPEGITTEEKINYMLEMRASGRNKVQDYADKFGVQFFPGEKPWGQ KVDIIM
α13 l α14 m α15 α15b
PCATQNEIDEHDAELLIKHGCQYVVEGANMPSTNEAIH KYNKAGIIYCPGKAANAGGVAV 419
PCATQNDVDLEQAKKIVANNVKYYIEVANMPTTNEALRFLMQQPNMVVAPSKAVNAGGVLV
α15b α16 α17
SGLEMTQNRMSLNWTREEVRDKLERIMKDIYDSAMGPSRRYNV DLAAGANIAGFTKVAD 478
SGFEMSQNSERLSWTAEEVDSKLHQVMTDIHDGSAAAAERYGLGYNLVAGANIVGFQKIAD
AVKAQGAV 486
AMMAQGIAW

that is conserved among all GDHs, whereas domain two contains the characteristic βαβ dinucleotide-binding fold ($\beta_g\alpha_{10}\beta_h$) responsible for $NAD(P)^+$ binding (Rice et al., 1987). This fold contains the consensus GXGXXA/G (residues 276 to 281) nucleotide binding fingerprint. The preference for alanine (281) as the last residue observed in the *C. sorokiniana* sequence (Fig. 18) is indicative of $NADP^+$-specific dehydrogenases, whereas a glycine residue is typical in NAD^+-specific enzymes (Baker et al. 1992). The two domains are separated by a deep cleft that in the tertiary structure of the subunit forms a catalytic pocket to bring the two substrates in close proximity (Baker et al., 1992). The lysine 234 and glutamate 423 (Fig. 18) are predicted to form a salt bridge that is responsible for stabilizing the tertiary structure of the NADP-GDH subunits (Britton et al., 1992). Additionally, regions of domain one (α_1-α_5,β_a-β_b,α_{17} [Fig. 18]) were shown to play a critical role in the dimerization and trimerization processes that lead to the catalytically active hexameric GDH (Baker et al., 1992).

Comparison of the structural predictions of the α-subunit and β-subunit revealed the α-subunit possesses an additional amino-terminal α-helical domain that is lacking in the β-subunit (Fig. 18). Comparison of NADP-GDH α- and β-subunit amino acid sequences to the greater than 35 GDH sequences in the protein data bases revealed that the *C. sorokiniana* NADP-GDH α- and β-subunits possessed an additional 41 and 30 unique amino-terminal residues,

respectively. Comparison of the unique regions to all other proteins in the data bases yielded no evidence of a conserved functional domain. However, visual inspection revealed that the residues 33 to 40 (GTEGLVHG or GXXGXXXG [Fig. 18]) of the unique region form a glycine-rich turn that closely resembles the motif GXGXXG of the nucleotide-binding fingerprint.

Comparison of the Induction Patterns of the NADP-GDH Antigens, Activities, and mRNAs in 29 mM Ammonium Medium

The cellular levels of the NADP-GDH antigens, enzyme activities, and mRNAs were measured throughout a 240 min induction in ammonium medium to determine if a correlation exists between a specific NADP-GDH mRNA and a specific activity or subunit type. Synchronous nitrate-cultured *C. sorokiniana* cells were transferred to 29 mM ammonium medium and cultured in continuous light for 240 min according to Cock et al. (1991). Samples were collected at various time intervals throughout the induction for the analysis of enzyme activity, antigen, and mRNA.

During the 240 min induction period, culture turbidity increased 1.87 fold (Fig. 19); however, the culture cell number remained constant at 2.89 x 10^7 cells per mL. Total soluble protein showed an initial decrease in the first 20 min followed by a rapid linear increase between 40 and 140 min that slowed at 240 min (Fig. 20).

The pattern of NADP-GDH antigen accumulation was examined by use of a western blot immunodetection procedure.

Figure 19. Increase in culture turbidity of *Chlorella* cells cultured for 240 min after the addition of 29 mM ammonium. At zero time, ammonium was added to synchronous daughter cells and the culture was illuminated. Cell number remained constant throughout the induction time period.

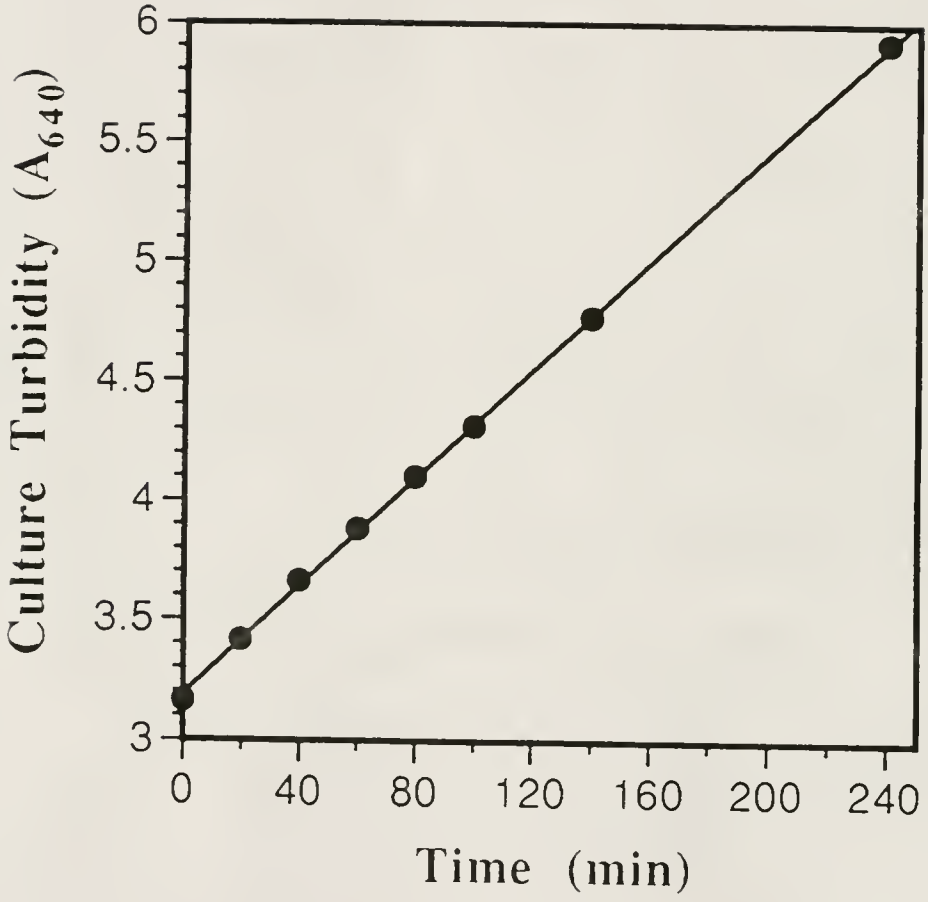
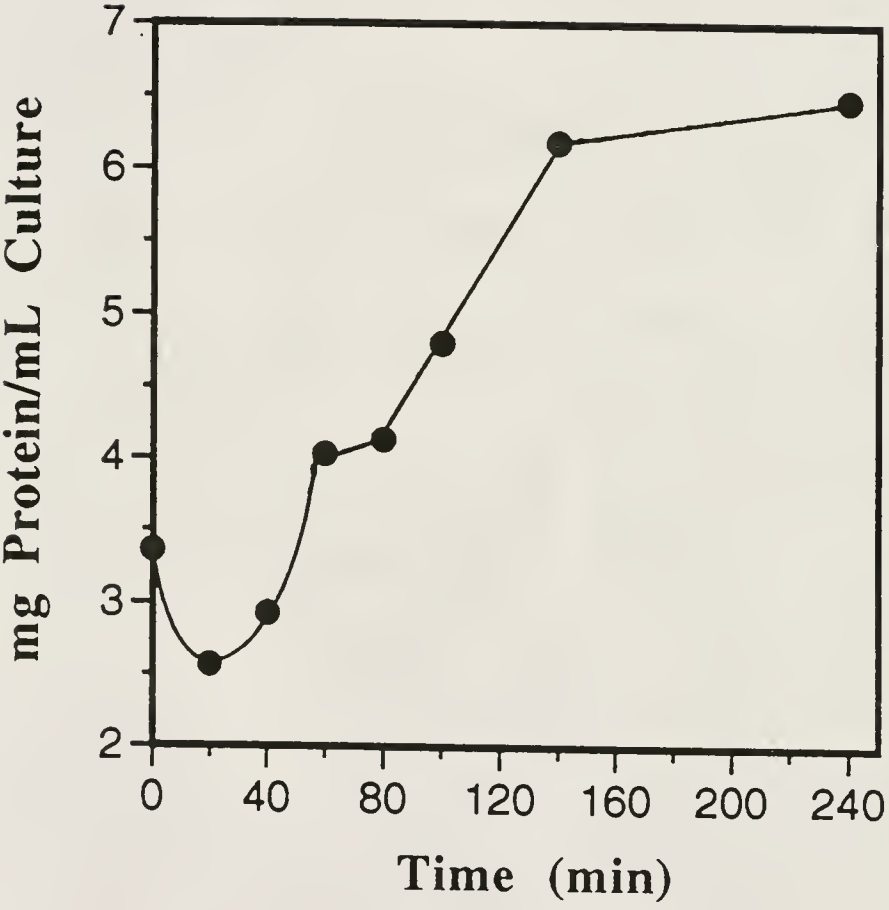

Culture Turbidity (A_{640})
6
5.5
5
4.5
4
3.5
3
0
40
80
120
160
200
240
Time (min)

Figure 20. Pattern of the total soluble protein in synchronized daughter cells induced for 240 min in 29 mM ammonium medium. At zero time, ammonium was added to synchronized cells and the culture was illuminated. Culture samples were collected at various time intervals, ruptured, and the clarified supernatants were analyzed according to Bradford (1976) for total protein content.


mg Protein/mL Culture
7
6
5
4
3
2
0
40
80
120
160
200
240
Time (min)

The NADP-GDH β-subunit (52.3 kD) was detected at T_0 and increased for the first 40 min followed by a gradual decrease that continued for the remainder of the induction period (Fig. 21, Fig. 22). The α-subunit (53.5 kD) was first detected at 20 min and gradually increased for the first 80 min followed by a marked increase that continued for the remainder on the induction period (Fig. 21, Fig. 22). The transition from the β-subunit being the prominent species to the α-subunit being prominent occurred between 60 and 80 min.

The NADPH-GDH (aminating) and the $NADP^+$-GDH (deaminating) activities were monitored throughout the 240 min induction period. Both the NADPH- and $NADP^+$-GDH activities showed an initial lag of 20 and 40 min, respectively (Fig. 23). The NADPH-GDH activity increased rapidly for the first 80 min and declined sharply at 100 min before increasing at 240 min. The $NADP^+$-GDH activity accumulated at a linear rate throughout the induction after an initial lag (Fig. 23). The NADPH-GDH:$NADP^+$-GDH activity ratio and the α:β subunit ratio were calculated to determine if the α- or β-subunit ratio was correlated with a particular GDH activity (Table 6).

Table 6. Ratios of NADP:$NADP^+$ activities and α:β subunits

Time (min)	Ratio NADPH:$NADP^+$ GDH Activity	Ratio α:β Subunit
0	2.87	0.28
20	2.96	0.58
40	3.81	0.49
60	4.51	0.80
80	3.49	1.57
100	2.73	8.74
140	1.61	11.23
240	1.13	34.79

Figure 21. Patterns of accumulation of NADP-GDH antigens in illuminated cells cultured in 29 mM ammonium medium for 240 min. At zerp time, ammonium was added to synchronous daughter cells and the culture was illuminated. The proteins in equal volumes of homogenates, from cells harvested at the various time points, were resolved by SDS-PAGE and transferred to nitrocellulose. The resulting blot was immunoreacted with polyclonal rabbit anti-NADP-GDH antibodies labelled with ^{125}I-Protein A. Lanes are labelled with corresponding time points. The positions of the α-subunit (53.5 kD) and β-subunit (52.3 kD) are noted.

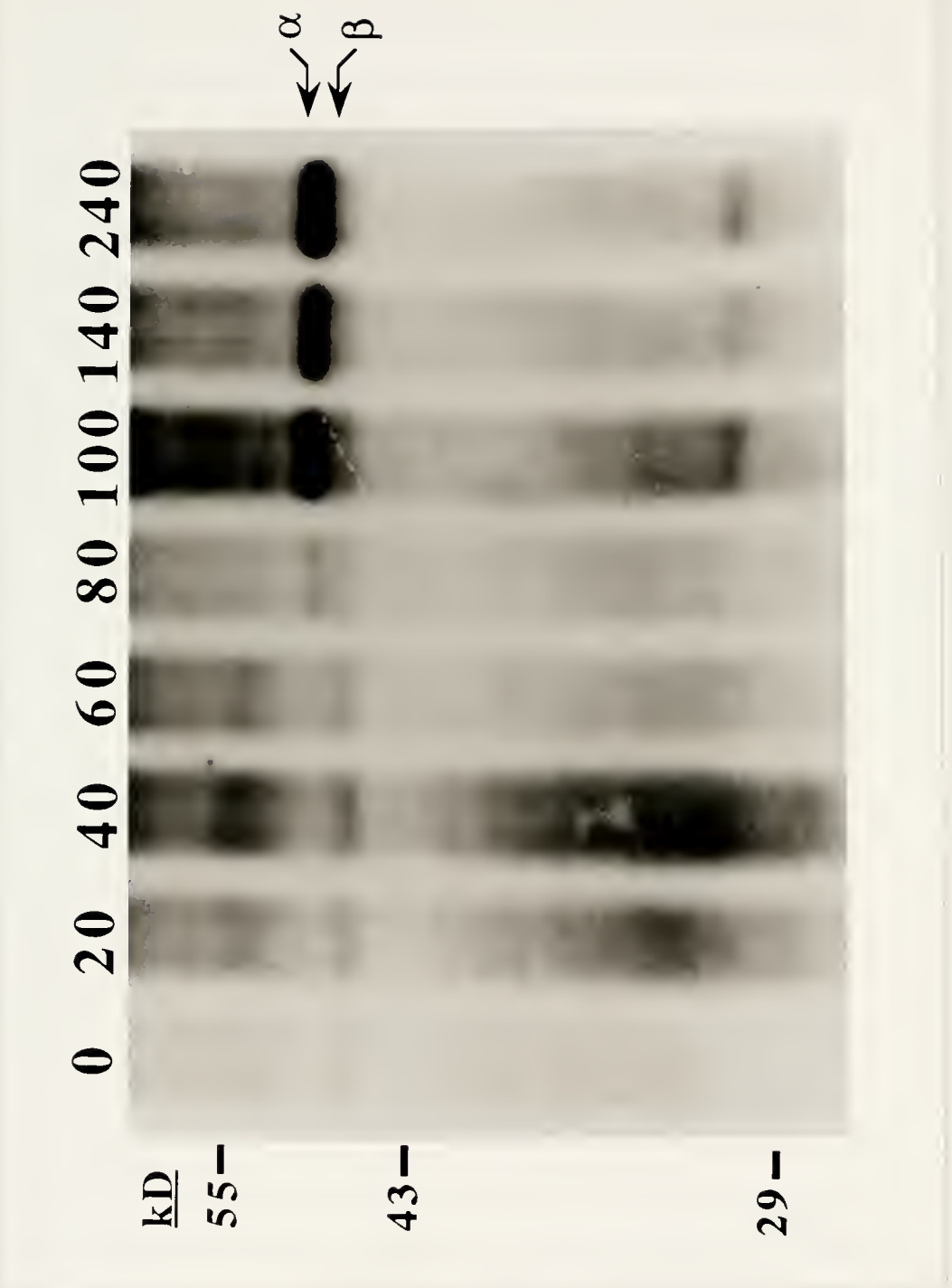

0 20 40 60 80 100 140 240
α
β
kD
55
43
29

Figure 22. Patterns of accumulation of NADP-GDH antigens in illuminated cells cultured in 29 mM ammonium medium for 240 min. At zero time, ammonium was added to synchronous *C. sorokiniana* daughter cells and the culture was illuminated. Autoradiographs of Western blots (Fig. 21) were analyzed by laser densitometry to determine the relative levels of the NADP-GDH α-subunit (●) and β-subunit (○) throughout the 240 min induction period.

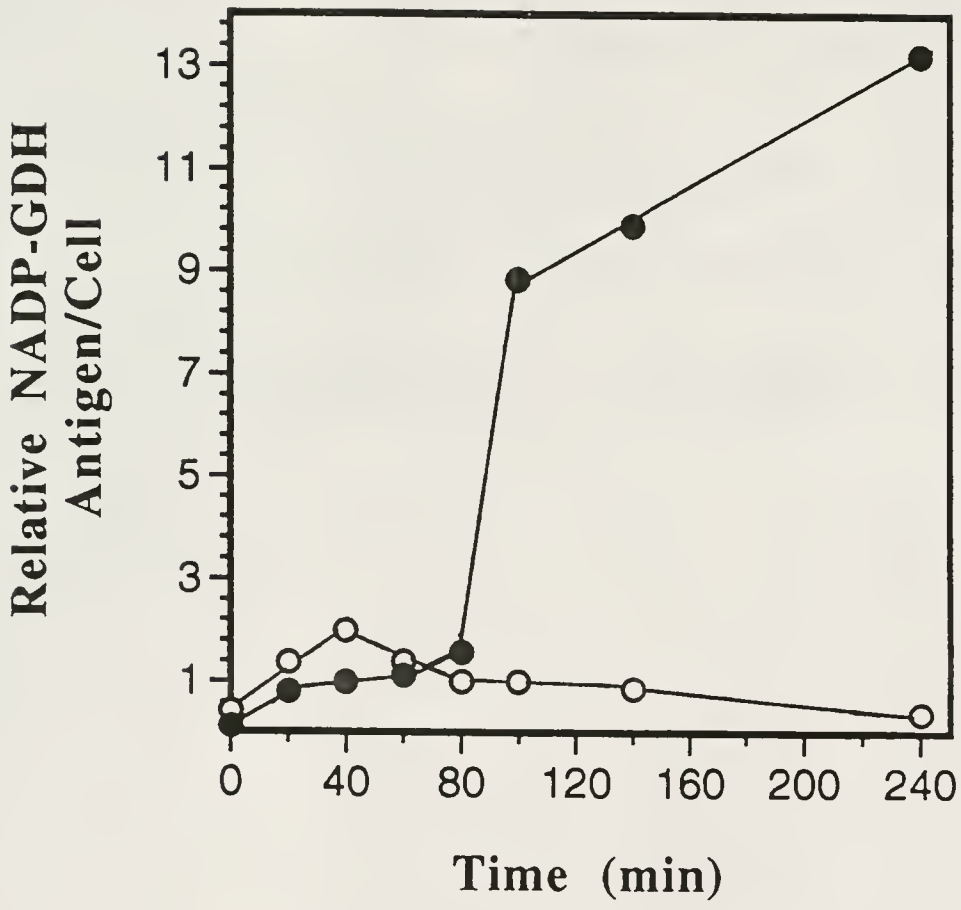
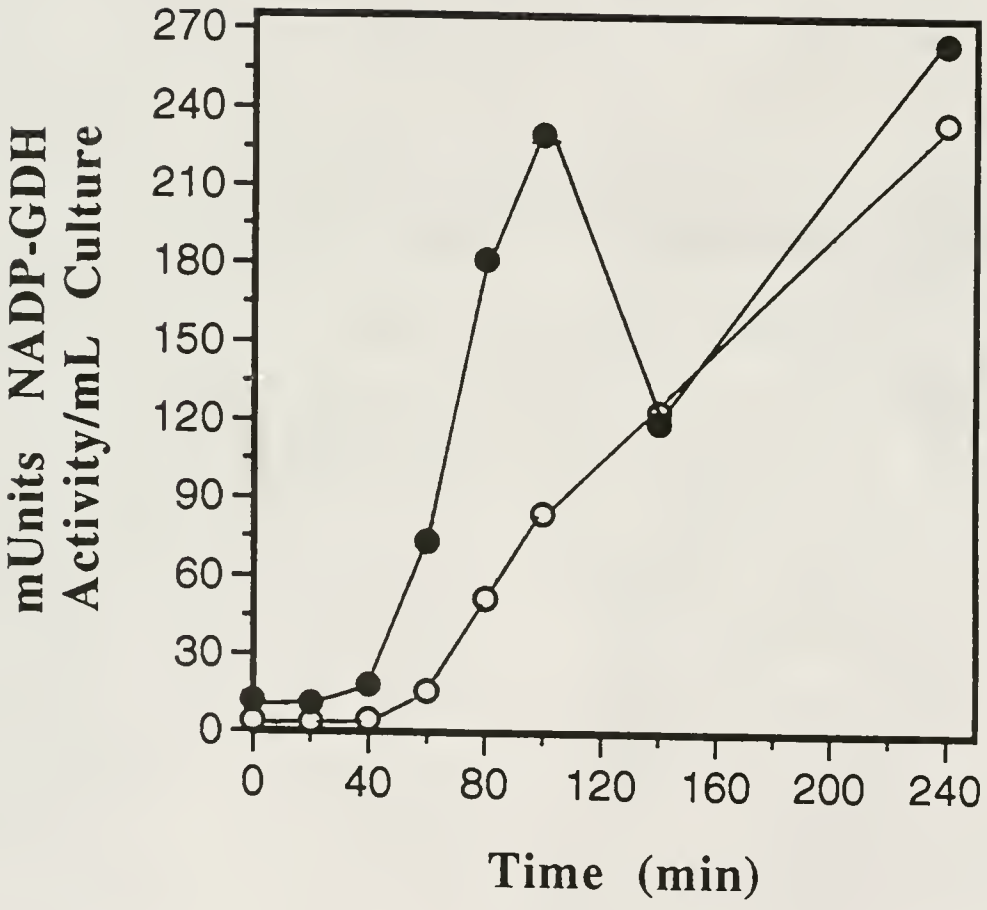

Relative NADP-GDH
Antigen/Cell
13
11
9
7
5
3
1
0
40
80
120
160
200
240
Time (min)

Figure 23. Pattern of NADP-GDH activities in homogenates of synchronous *C. sorokiniana* cells cultured for 240 min in 29 mM ammonium medium in continuous light. Aliquots of clarified homogenates, from cell collected at various time intervals, were analyzed spectrophotometrically for both aminating (●) and deaminating (○) NADP-GDH activities.

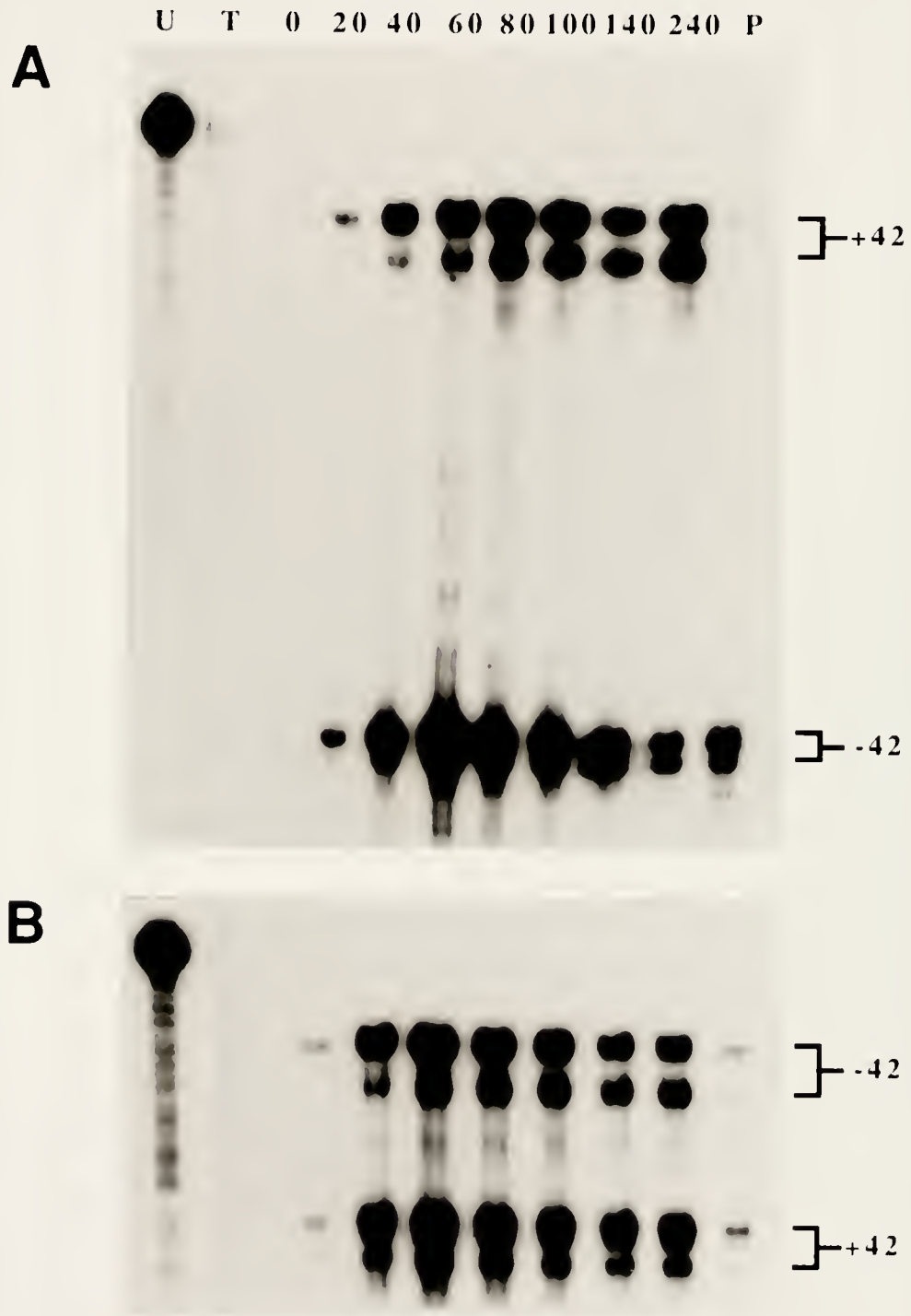

mUnits NADP-GDH
Activity/mL Culture
270
240
210
180
150
120
90
60
30
0
0
40
80
120
160
200
240
Time (min)

The peak NADPH:$NADP^+$-GDH ratio occurred at 60 min when the subunit ratio showed the β-subunit to be the prominent NADP-GDH antigen, whereas the α-subunit was the prominent form when the NADPH:NADP+-GDH ratio was the lowest.

RPA of total RNA from each time point was used to elucidate the relative induction patterns of the +42 nt and -42 nt NADP-GDH mRNAs. Total RNA from each time point, containing equal amounts of $poly(A)^+$ mRNA, was hybridized with an antisense protecting probe corresponding to the 5'-VR of the +42 nt mRNA, 5'-VR of the -42 nt mRNA, HCR, or 3'-UTR. The hybridized products were treated with a ribonuclease mixture, and the resulting products were analyzed on a 5% sequencing gel.

Two unique protected regions (Fig. 24A, B) corresponding to the 5'-VR were detected throughout the induction period with the +42 nt 5'-VR probe (ca. 367 and 170 nt) and -42 nt 5'-VR probe (ca. 309 and 200 nt). A single protected region (Fig. 24C, D) was detected throughout the induction with the probes corresponding to the HCR (ca. 154 nt) and 3'-UTR (ca. 388 nt). Each protected region occurred as a doublet, separated by 4 to 11 nt, that resulted from the cleavage of the protecting fragment by RNase A (cleaves after C, U) or RNase T_1 (cleaves after G) in the RNase mixture. To verify the doublet represented a single protected region, RNase I, a ribonuclease that cleaves in a nucleotide independent manner, was utilized in a single set of RPAs with the -42 nt 5'-VR protecting probe. Two protected regions, occurring as single

Figure 24. Ribonuclease protection analysis of the NADP-GDH mRNAs synthesized in synchronous *C. sorokiniana* cells throughout a 240 min induction period in 29 mM ammonium medium. Total RNA, containing equal amounts of poly(A)$^+$ RNA, isolated from cells collected at various time intervals, was hybridized with antisense RNA probes corresponding to the 5'-VR of the +42 bp mRNA (A), 5'-VR of the -42 bp mRNA (B), HCR (C), and 3'-UTR (D). The resulting hybrids were treated with a RNase mixture and the RNase resistant fragments were resolved on a 5% sequencing gel and analyzed by phosphorimaging. A, +42 bp protected region: 373 nt, 362 nt (ca. 367 nt); -42 bp protected region: 172 nt, 167 nt (ca. 170 nt). B, -42 bp protected region: 311 bp, 306 nt (ca. 309 nt); +42 bp protected region: 202 nt, 198 nt (ca. 200 nt). C, HCR protected region: 155 nt, 152 nt (ca. 154 nt). D, 3'-UTR protected region: 392 nt, 381 nt (ca. 388 nt). Lanes U, unprocessed antisense probe; T, tRNA control hybridized with probe and Rnase treated; 0-240 min sample times; P, poly(A) RNA used in RACE-PCR.


U T 0 20 40 60 80 100 140 240 P
A
+42
-42
B
-42
+42

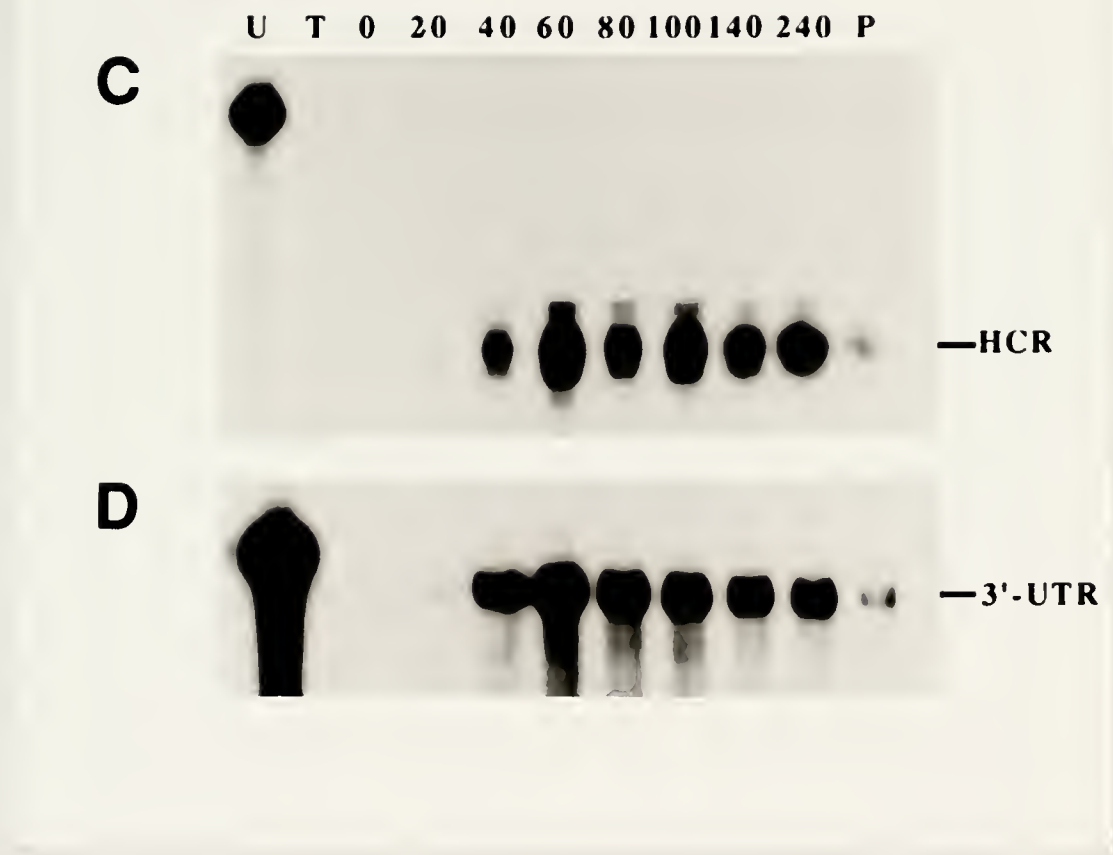
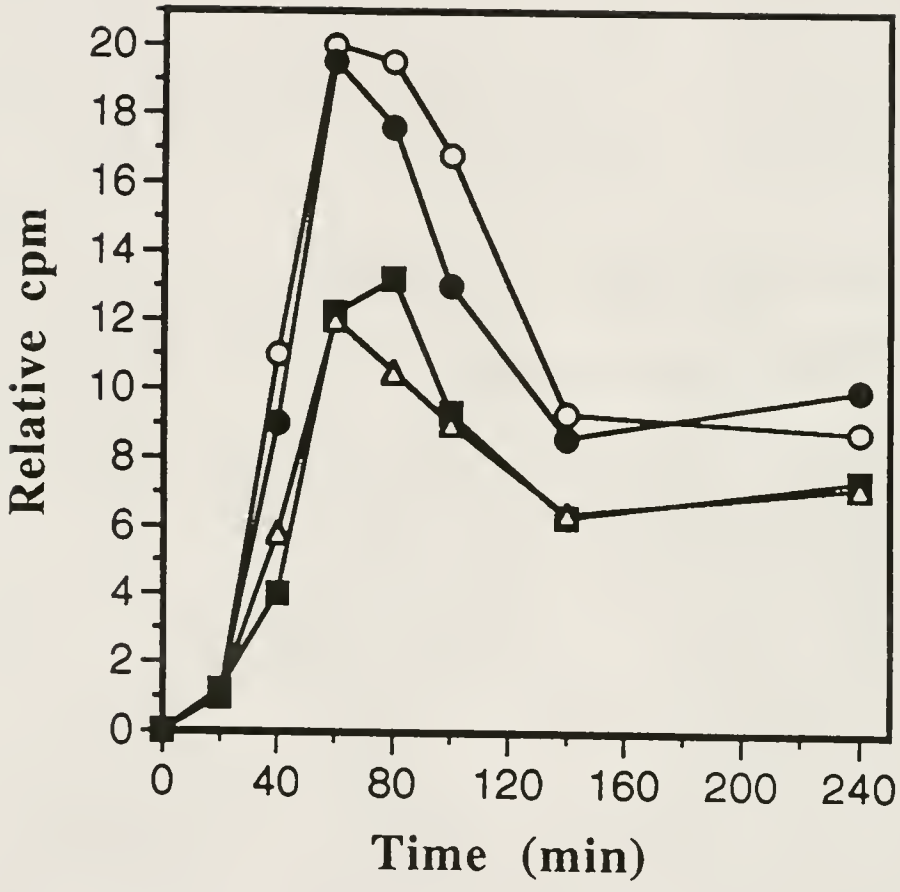


Figure 24 continued

fragments, were detected. The RNase I resistant fragments corresponded to identical regions detected as doublets with the RNase A-T_1 mixture (data not shown). Because of the quantity of units required and the expense of the RNase I, it was not feasible to perform all of the RPAs with this enzyme. The +42 nt 5'-VR probe yielded a protected region (170 nt) corresponding to the -42 nt mRNA (Fig. 24A) that was approximately 30 nt shorter than the protected fragment predicted by the cDNA sequence (200 nt). However, the reciprocal probe, -42 nt 5'-VR, yielded two protected regions of predicted size (309 and 200 nt). This paradox may reflect a probe-specific artifact due to the mis-incorporation of a nucleotide in the PCR generated +42 nt 5'-VR probe template, or a unique hybridization artifact.

The relative induction pattern of each protected region was determined by analysis on a phosphoimager. No NADP-GDH mRNA was detected with any of the probes in the T_0 sample. All four of the the protected regions showed a similar induction pattern for the first 140 min (Fig. 25). The protected region corresponding to the -42 nt NADP-GDH mRNA showed a decrease at 240 min that contrasted with an increase of the +42 nt mRNA (Fig. 25). The 3'-UTR probe, that hybridizes to both mRNAs, showed a peak mRNA abundance at 80 min in contrast to the 60 min peak observed for the other regions. RPA only detects RNA degradation in the region corresponding to the antisense probe and is not affected by degradation outside of this region. Therefore, the observed

Figure 25. Relative abundance patterns of NADP-GDH mRNA in cells induced in 29 mM ammonium medium. The abundance pattern of the +42 bp mRNA (●) and -42 bp mRNA (○) were determined by quantifying ^{32}P-labeled RNase resistant fragments resulting from differential digestion of the -42 bp 5'-VR RPA probe. The abundance patterns for total NADP-GDH mRNA was determined by quantifying ^{32}P-labeled RNase resistant fragments corresponding to the HCR (△) and 3'-UTR (■). The relative cpm of each protected fragment was quantified on a phosphorimager.


Relative cpm
20
18
16
14
12
10
8
6
4
2
0
0
40
80
120
160
200
240
Time (min)

shift may reflect the 5' to 3' degradation of eukaryotic mRNA reported by Brawerman (1993). The induction patterns observed with the HCR probe were equivalent to the pattern detected by Cock et al. (1991) by northern blot analysis.

Quantitative comparison of the relative abundances of the +42 nt mRNA and the -42 nt mRNA with respect to each other could not be easily accomplished with this procedure. Since the antisense protecting probes were radiolabeled with a single nucleotide (dCTP), the specific activity was affected by the number of cytosine residues remaining in the processed product. The processing of the -42 nt protecting fragment hybrized to the +42 nt mRNA preferentally removed regions rich in cytosine that artificially lowered the cpm emmitted by this protected region relative to the -42 nt mRNA protected fragment.

RT-PCR Analysis of the NADP-GDH mRNAs

To determine if the +42 nt and -42 nt mRNAs differ in any region other than the 5'-VR and to quantify the relative abundance of each mRNA, mRNA from various time points of the 240 min 29 mM ammonium induction was analyzed by RT-PCR (Kawasaki, 1990). Total RNA, containing equal amounts of poly(A)$^+$ RNA, from each time point was amplified using a series of primers spanning the entire length of the NADP-GDH mRNAs. The final PCR products were analyzed on ethidium-stained agarose gels and compared to control reactions that utilized cDNA clones bearing the region(s) of interest as PCR

templates. Photographic negatives of the ethidium-stained gels were quantified using a laser densitometer.

RT-PCR analysis revealed that the +42 nt and -42 nt NADP-GDH mRNAs differed only in their 5'-VRs (Fig. 26). A single RT-PCR product was detected for all other regions of the mRNAs (Fig. 26C-F). The PCR products were identical to the sizes predicted from the cDNA sequences and to the plasmid DNA control products. No PCR products were detected in control mRNA samples in which no reverse transcriptase was added, verifying the observed products did not result from amplification of contaminating DNA. NADP-GDH mRNA was not detected in the T_0 sample and the relative induction pattern of each region was equivalent to those determined by RPA (Fig. 25).

Quantitative comparison of the two NADP-GDH mRNAs revealed the relative abundance patterns of the two mRNAs (Fig. 26A,B) differed significantly (Fig. 27). The +42 nt mRNA was approximately three times more abundant than the -42 nt mRNA throughout the induction time course. Both mRNAs showed a similar induction pattern from 20 to 140 min; however, the +42 nt mRNA showed an increase at 240 min that contrasted with a decrease of the -42 nt mRNA (Fig. 27). This observation is in agreement with the contrasting shift observed for the two mRNAs at 240 min using the RPA (Fig. 25).

Figure 26. RT-PCR analysis of the NADP-GDH mRNAs synthesized in synchronous *C. sorokiniana* cells throughout a 240 min induction period in 29 mM ammonium medium. Total RNA, containing equal amounts of poly(A)$^+$ RNA, isolated from cells collected at various time intervals, was amplified using a series of primer pairs spanning the entire length of the NADP-GDH mRNAs. The resulting PCR fragments were resolved on agarose gels and the molecular weight of the products were determined by comparison to commercial standards (M). A, 147 bp and 105 bp. B, 311 bp and 269 bp. C, 464 bp. D, 155 bp. E, 760 bp. F, 745 bp. The RT-PCR products from each time point (0 to 240 min) were compared to products amplified from cDNA plasmid templates bearing the regions of interest (control).

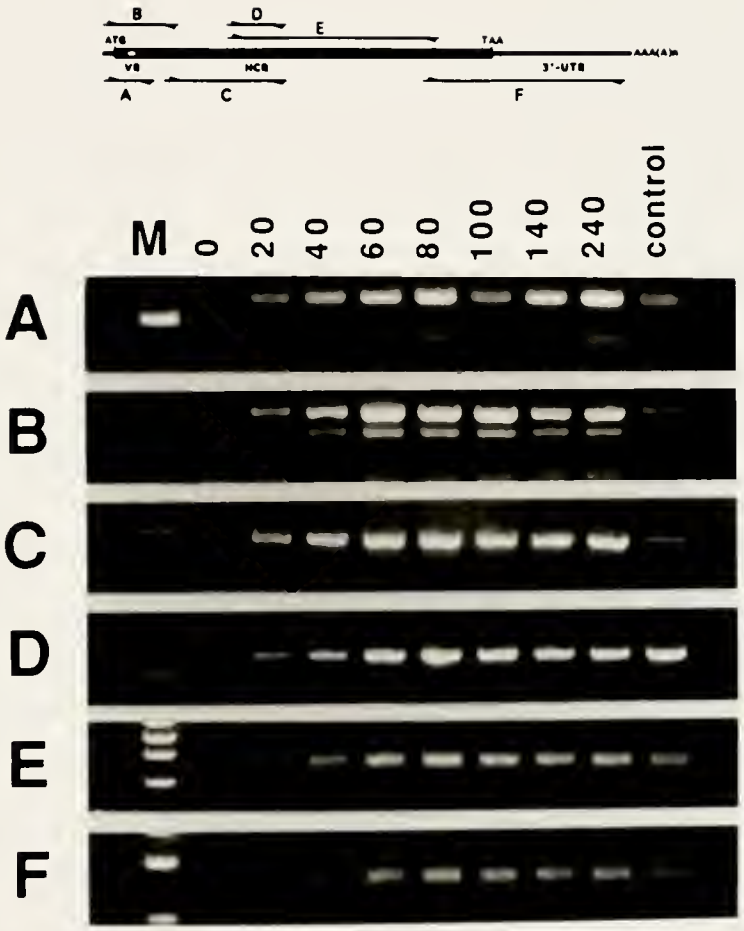
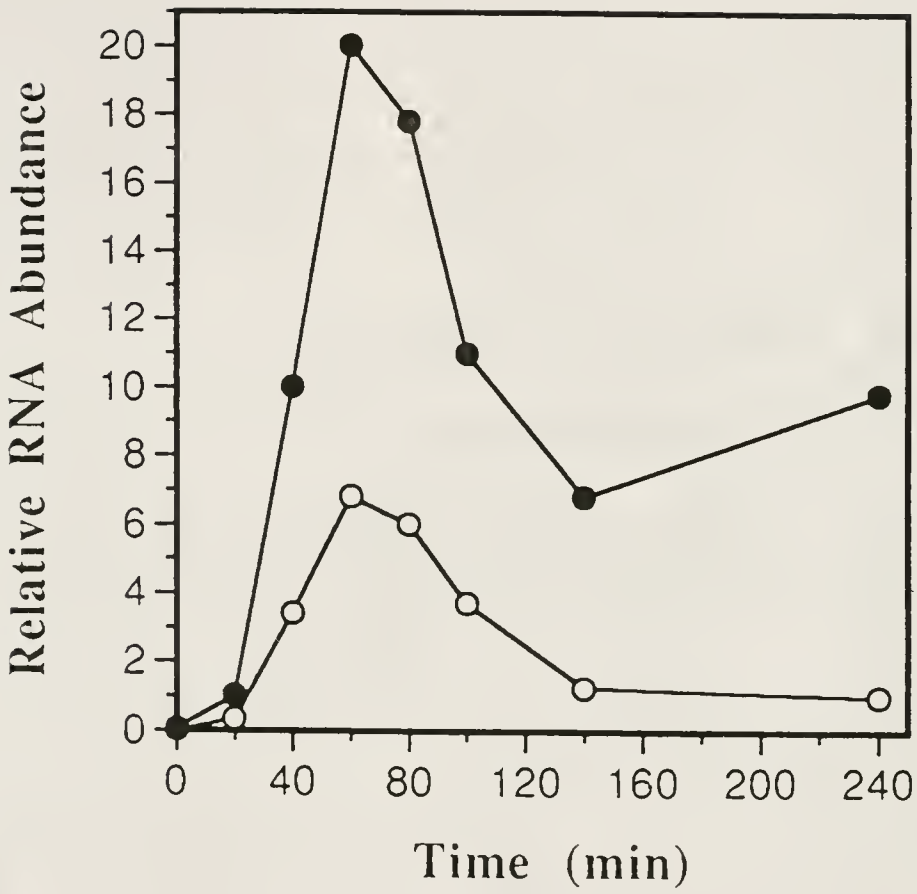

B
D
E
ATG
TAA
AAA(A)n
V5
HCR
3'-UTR
A
C
F
M
0
20
40
60
80
100
140
240
control
A
B
C
D
E
F

Figure 27. Relative abundances of the NADP-GDH mRNAs synthesized in synchronous *C. sorokiniana* cells throughout a 240 min induction period in 29 mM ammonium medium. RT-PCR products corresponding to the +42 bp (●) and -42 bp (○) mRNAs, derived from primer pairs spanning the 5'-VR (Fig. 26 A,B), were quantified by laser desitometry to determine the relative abundances of each specific NADP-GDH mRNA during the induction period.


Relative RNA Abundance
20
18
16
14
12
10
8
6
4
2
0
0
40
80
120
160
200
240
Time (min)

DISCUSSION

The results presented in this study reveal the *C. sorokiniana* nuclear genome possesses a single NADP-GDH gene that is transcribed to yield, via alternative pre-mRNA splicing, two NADP-GDH mRNAs. Cock et al. (1991) provided Southern blot and genomic cloning evidence that was consistent with the existence of a single NADP-GDH gene in the *C. sorokiniana* genome. However, the significance of these findings was limited by the fact that none of the NADP-GDH cDNA clones isolated possessed complete 5'-terminal sequences and many lacked a 3' terminus. The lack of terminal sequences in the cDNAs prevented the researchers from determining whether the NADP-GDH gene yields a single or multiple mRNAs that encode the NADP-GDH α- and β-subunits.

In this study, experimental evidence was obtained for two NADP-GDH mRNAs that are identical except for the presence of a 42 nt insert located in the 5'-coding region of the longest mRNA. The full-length NADP-GDH mRNA sequences were assembled by combining overlapping regions of identical sequence of various cDNA clones (Figs. 1,3). Several lines of evidence support the inference that the two NADP-GDH mRNA sequences actually exist *in vivo*.

RPA of total cellular RNA isolated from cells synthesizing both the α- and β- subunits detected a single

RNase resistant region with the HCR and 3'-UTR probes, whereas two resistant regions were detected with both the +42 bp and -42 bp 5'-VR probes (Fig. 24). These results provide strong evidence that the two unique 5'-VRs do exist *in vivo* and are not an artifact of the 5' RACE-PCR cloning. RT-PCR analysis of mRNA isolated from cells synthesizing both subunits, using overlapping primer pairs that span the entire lengths of the NADP-GDH mRNAs, yielded a single PCR product for each region of amplification downstream of the 5'-VR (Fig. 26 C-F). Primer-pairs, designed to anneal to regions proximal and distal to the 5'-VR, yielded two PCR products differing by 42 bp (Fig. 26 A,B). The molecular weights of the mature α- and β-subunits deduced from the combined cDNA clones were nearly identical to the molecular weights of the *in vivo* subunits determined by SDS-PAGE. These results support the authenticity of the +42 bp and -42 bp NADP-GDH mRNA sequences.

Although both mRNA sequences could be accounted for in the previously determined nuclear NADP-GDH gene sequence, the possiblity remained that two closely related NADP-GDH genes might exist that differ only at their 5'-termini corresponding to the 5'-VR of the mRNAs. Southern blot analysis using the 5'-VR, HCR, and 3'-VR probes yielded hybridization patterns consistent with patterns predicted by the single NADP-GDH gene sequence (Fig. 12). In addition, allele-specific PCR analysis of *C. sorokiniana* genomic DNA yielded single PCR products corresponding to the 5'-VR, HCR,

and 3'-UTR identical in size to corresponding products amplified from the NADP-GDH genomic clones (Fig. 13). These results are consistent with the existence of only a single NADP-GDH gene in the *C. sorokiniana* nuclear genome.

Comparison of the NADP-GDH +42 and -42 bp cDNA sequences to the NADP-GDH gene sequence revealed the mRNA sequences span a 7178 bp region of the *C. sorokiniana* nuclear genome (Fig. 19). The +42 nt mRNA is divided over 22 exons ranging in size from 18 to 550 bp, whereas the -42 nt mRNA is divided over 23 exons ranging in size from nine to 550 bp. The intron/exon splice junctions are highly conserved and closely match the consensus sequences identified for constitutively spliced exons in higher plants and animals (Brown, 1986; Hawkins, 1988).

The 42 bp intron (auxon), which is alternatively spliced to yield the two NADP-GDH mRNAs, lacks the consensus splice-site elements. Since the splice-site junction sequences of the 42 nt auxon lack conserved sequence elements, there might be a different recognition involved in the splicing mechanism. The splice elements involved in alternative RNA splicing often do not appear to differ significantly from constitutively-spliced elements (Breitbart et al., 1987a), though in some reported cases extreme variants of the sequence and arrangement of the elements influence alternate splice-site utilization. In addition to *cis*-elements, regulation of alternate splice-site utilization also involves differences in cellular *trans*-factors as evidenced by

developmentally and cell-specific regulated splicing (Breitbart et al., 1987b). The retention or removal of the 42 bp auxon might be determined by specific *trans*-factors that are only present under certain physiological conditions. The additional *trans*-factors could simply be variants of the snRNPs associated with the constitutive sliceosome complex (Smith et al., 1989). In mouse and *Xenopus*, multiple U1 snRNAs associated with U1 snRNP have been isolated and characterized and have been shown to be developmentally regulated (Lund et al., 1985; Lund and Dahlberg, 1987). Since the U1 snRNP is responsible for 5' splice-junction recognition, it has been proposed that sequence differences in the multiple U1 snRNA allow for recognition of alternative 5' splice-sites in developmentally regulated alternative RNA splicing. Heterogeneity in snRNAs associated with other snRNPs of the spliceosome complex have also been implicated in alternate splice-site utilization (Smith et al., 1989). The possiblity cannot be overlooked that the 42 bp auxon could be alternately removed by a unique processing mechanism that does not involve the spliceosome complex.

The small size of the 42 nt auxon and 9 nt exon is smaller than any intron or exon reported for a plant gene. In addition to specific *cis*-elements and *trans*-factor requirements for RNA splicing, there also appears to be minimal distance requirements that reflect steric constraints for the binding of splicing factors to the pre-mRNA (Chabot and Steitz, 1987). In mammalian genes, a minimal intron

length of 66 nt has been determined; however, introns as small as 31 nt (Craig et al., 1989) and 43 nt (DeWet et al., 1987) have been shown to be effectively spliced in nematodes and *Photinus*, respectively. Therefore, there is no biochemical precedent to preclude the processing of the 42 nt intron. There appear to be no restrictions on exon length, as exons of 3 nt (Santoni et al., 1989) and 6 nt (Cooper and Ordahl, 1985) have been identified in mammals.

To identify the putative NADP-GDH promoter region, the 5'-terminal sequences of the NADP-GDH mRNAs were compared to the GDH gene sequence. Comparison of the sequences identified the transcriptional initiation site. An additional 1285 bp of genomic DNA sequence was identified upstream of the initiation site. Since the induction rate of the NADP-GDH isoenzymes is influenced by various carbon and nitrogen compounds, it is anticipated the NADP-GDH promoter might have cis-elements sensitive to these various molecular signals. The putative NADP-GDH promoter region (Fig. 11) does not possess a consensus TATA box nor CCAAT box normally located at position -25 to -35 bp and -50 to -100 bp, respectively. No positive or negative cis-regulatory elements common to other previously identified plant promoter elements were detected (Kuhlemeier, 1992; Katagui and Chua, 1992). The promoter region of the *Chlorella kessleri* H+/hexose cotransporter gene (Wolfe et al., 1991), the only other *Chlorella* gene published to date, also lacks these *cis*-regulatory elements. Comparison of the *C. kessleri* promoter

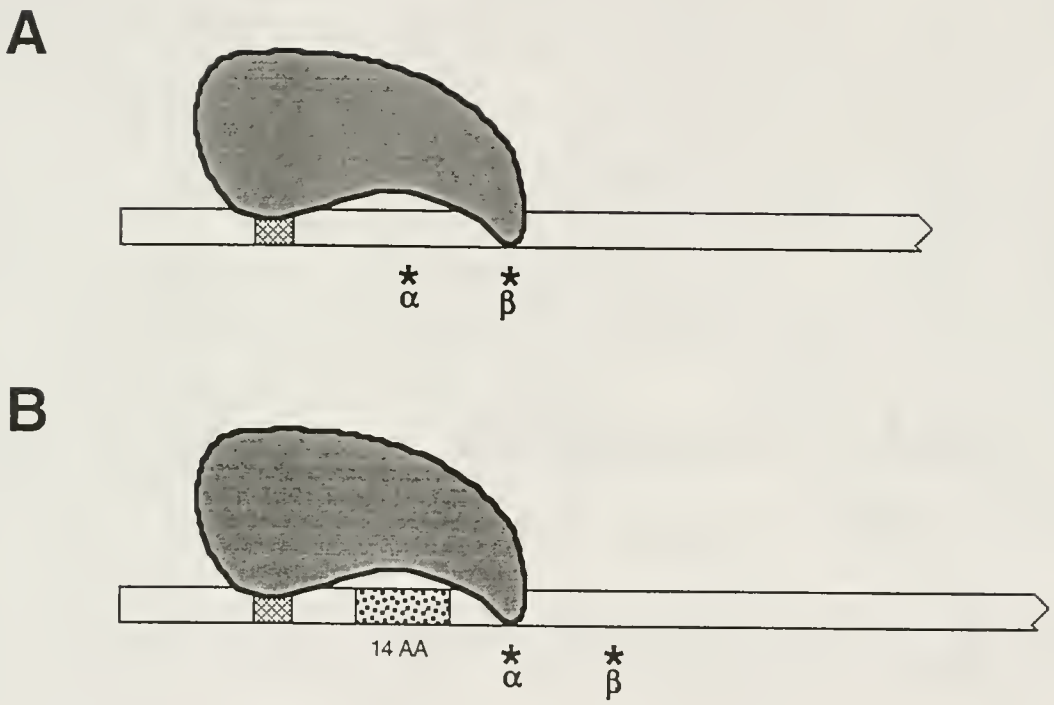
region to the putative NADP-GDH promoter region revealed no common upstream elements. Although no common promoter elements were identified in the 1285 bp upstream sequence, the evidence presented herein supports the identification of the transcriptional start site. The 5'-terminal sequences of the NADP-GDH mRNAs possessed a 32 bp pyrimidine rich (89%) 5'-UTR and a translation initiation sequence indicative of eukaryotic mRNAs (Kozak, 1984). In addition, the presence of unique 5' guanine residues in the 5' RACE-PCR clones provides a definitive means for the identification of the 5'-capping points of the two NADP-GDH mRNAs (Bahring et al., 1994). The identification of the transcriptional start-site and putative promoter region should allow analysis of sequence elements involved in the metabolic regulation of transcription of the NADP-GDH gene by *in vivo* footprinting.

The physiological significance of the 42 nt insert in the 5'-coding region of the +42 bp NADP-GDH mRNA is unclear (Fig. 6). The 42 nt insert or the lack thereof could potentially influence translational efficiency, mRNA turnover, precursor-protein import, or precursor processing by the SPP. Analysis of the secondary structural predictions of the two NADP-GDH precursor proteins indicates that the 42 nt insert introduces 14 amino acids residues that alter the structural characteristics of the transit peptide domain (Figs. 7,8). Transit peptide sequences differ considerably from one another even within a single organism and no strong secondary structural requirements seem to be necessary for

proper import or processing. Although the structural and sequence determinants for precursor-protein processing have not been well established, alterations in the carboxy-terminal regions of the transit peptide have been shown to influence processing (Smeekens et al., 1990). Therefore, the additional amino acids or secondary structure may influence processing of the precursor protein by the SPP.

One possiblity is the insertion of the additional amino acid residues influences the location of the SPP binding site in relation to the two SPP cleavage sites (Fig. 28). If the polypeptide lacks the 14 amino acid insert, the SPP cleavage domain might be placed in close proximity to the β-subunit cleavage site and yields the mature β-subunit (Fig. 28A). The presence of the 14 amino acid residues in the larger polypeptide might shift the downstream cleavage sites in relation to the SPP binding site and position the SPP cleavage domain in contact with the α-cleavage site, thus yielding the mature α-subunit (Fig. 28B). The possibility also exists that if the insert does influence processing it may do so by creating or eliminating a cleavage recognition domain. The possibility remains that the α- and β-subunits arise via differential processing of the precursor proteins by two different SPP; however, only a single general SPP has been identified in chloroplasts of all plants analyzed to date (Musgrove et al., 1989). The single SPP has been shown, by *in vitro* processing analysis, to process all stromal proteins to authentic mature proteins.

Figure 28. Model for the regulation of the processing of the two NADP-GDH precursor proteins. A, The NADP-GDH precursor protein lacking the 14 amino acid insert is cleaved at the β-subunit cleavage site downstream of the SPP binding domain (crosshatched box). B, The larger NADP-GDH precursor protein possessing an additional 14 amino acid insert (stippled region) is cleaved at the α-subunit cleavage site, due to a shift in the position of the cleavage sites relative to the SPP binding site. Asterisks denote the α- and β-cleavage site as noted.


A
α
β
B
14 AA
α
β

There are several other possiblities of how the 42 nt insert might affect the regulation of the NADP-GDH gene expression; however, there is some precedent to support the model proposed in Figure 28. As will be discussed later, there is a correlation between the relative abundance of the +42 nt mRNA and the level the NADP-GDH α-subunit. It is also worth noting that the two precursor proteins differ by approximately the same number of amino acid residues and molecular weights as the mature α- and β-subunits, 14 amino acids (1.5 kD) and 11 amino acids (1.2 kD), respectively. The possibility also exists that the alternative processing of the 42 nt insert is a nuclear splicing event that has no influence on the subunit type encoded by the mRNA. Since the variable splicing does not greatly alter the primary sequences of the precursor polypeptides, the splicing may have no deleterious effect and therefore has not been subject to any strong selective pressures to eliminate the splicing event. By use of *in vitro* transcribed/translated precursor proteins, one should be able to determine unequivocally whether a given precursor is processed to the α- or β-subunit.

Results presented previously (Yeung et al., 1981; Bascomb and Schmidt, 1987) and in this study indicate that the NADP-GDH α- and β-subunits have a high amount of primary sequence identity. Both MAbs (Fig. 16) and polyclonal antibodies (Fig. 21) produced against the α-subunit are able to detect the β-subunit on immunoblots. Yeung et al. (1981)

showed that rabbit anti-NADP-GDH β-subunit antibodies immunoprecipitated the α-subunit. The molecular weights of the mature α- and β-subunits predicted from the nearly identical amino acid sequences deduced from the NADP-GDH cDNA clones (53.5 and 52.34 kD, respectively) is in close agreement with the *in vivo* molecular weights determined by SDS-PAGE (53.48 and 52,27 kD). Furthermore, the amino-terminal residues identified for the α-subunit overlapped with nine identical residues identified in the β-subunit amino-terminus. These results indicate that the α- and β-subunits are identical except for the additional 11 amino acid residue extension identified in the α-subunit amino terminus (Fig. 18).

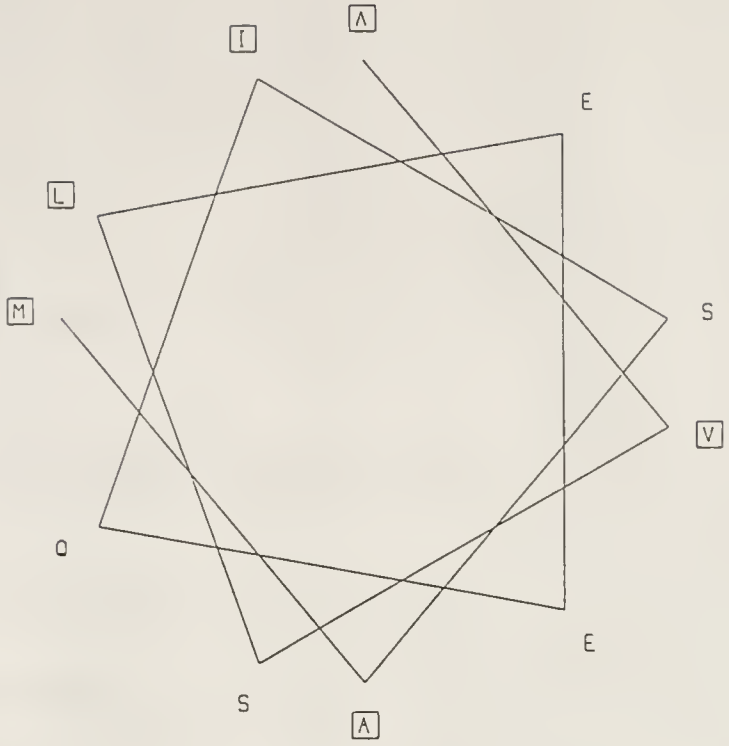
Comparison of the NADP-GDH α- and β-subunit sequences to the GDH sequences reported in the data bases revealed the two subunits possess an additional 41 and 30 amino-terminal residues that are unique to the *C. sorokiniana* NADP-GDH α- and β-subunits, respectively. Visual inspection revealed that residues 33 to 40 of the unique region (Fig. 18) form a glycine rich turn, GXXGXXXG, that closely resembles the motif, GXGXXG, of the dinucleotide-binding fingerprint. Although not an exact consensus, it is possible this region still interacts with pyridine dinucleotides (i.e. NADPH). A similar dinucleotide-binding site, GXXGXXA, has been identified in porcine malate dehydrogenase and the tetrameric NADP-GDHs (Britton et al., 1992). This motif might not be sufficient for strong $NADP^+$/NADPH binding; however, it might

provide for a weak interaction such as would be required for the NADPH allosteric regulation observed for the *C. sorokiniana* NADP-GDH α-homohexamer (Bascomb and Schmidt, 1987).

In an attempt to explain the kinetic and biochemical differences reported previously between the α- and β-homohexameric forms of the NADP-GDH isoenzymes (Bascomb and Schmidt, 1987), a comparison was made between the structural elements of the α- and β-subunits (Fig. 18). Both the α- and β-subunits possess an additional α-helical domain (α'_2) upstream of the putative dinucleotide-binding fingerprint (Fig. 18). The additional 11 amino-terminal residues of the α-subunit form a second α-helical domain (α'_1) not found in the β-subunit. Helical wheel projections of the two helical domains reveal that both domains possess a hydrophobic and hydrophilic face (Fig. 29). The hydrophobic faces of the α-helical domains may implicate these helicies as being important in subunit-subunit contacts, since the downstream α_1 helix common to all hexameric GDHs has been shown to be involved in subunit dimerization and trimerization (Baker et al., 1992). Comparison to the three-dimensional structure of the *C. symbiosum* GDH revealed the additional helical domains would be predicted to lie in the large central core formed in the assembled hexamers (Fig. 30), thus exposing the hydrophilic faces to the central cavity. In the non-allosterically regulated *C. symbiosum* GDH, all contacts between subunits involve domain one structures. However, the

Figure 29. Helical wheel projections of the unique *C.sorokiniana* NADP-GDH amino-terminal helical domains. A, Helical wheel projection of the α'_1 helix of the α-subunit. B, Helical wheel projection of the α'_2 helix identified in both the α- and β-subunits. Boxed residues identify hydrophobic amino acids.

A



B

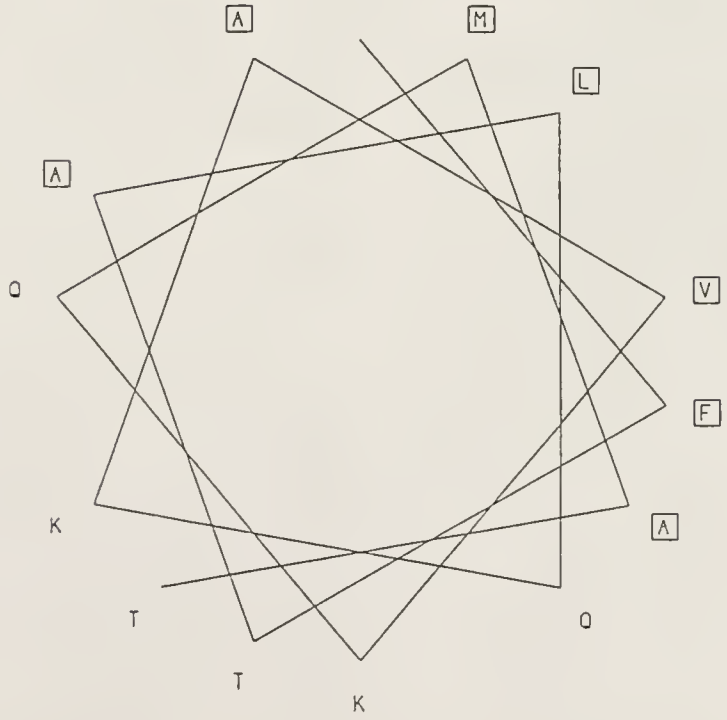
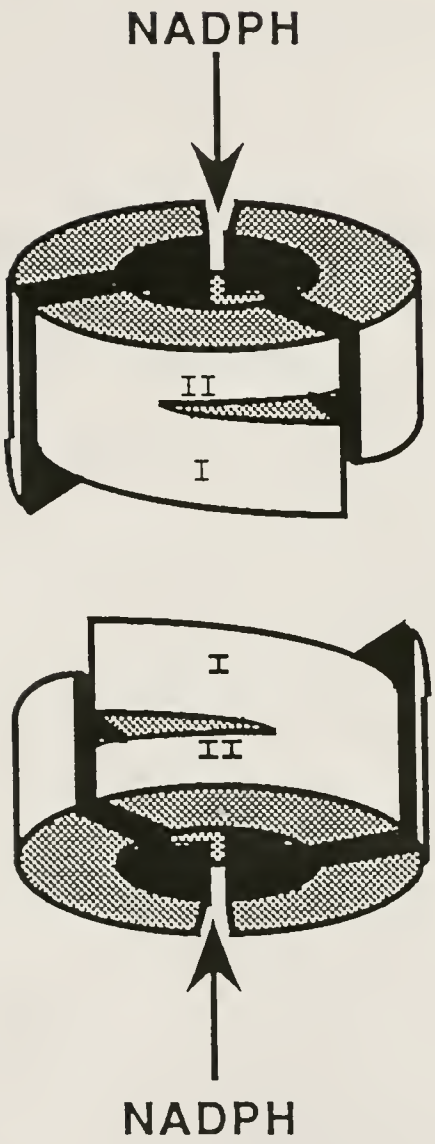


Figure 30. Diagramatic representation of the assembled hexameric NADP-GDH. Arrows denote the large central core where the putative dinucleotide binding-domain and the α'_1 and α'_2 helical domains are proposed to interact with the NADPH ligand. Dimerization and trimerization contacts involve domain one contacts. Diagram after Baker et al. (1992).


NADPH
II
I
I
II
NADPH

vertebrate GDHs possess approximately 50 carboxy-terminal resides proposed to be involved in domain two contacts (Baker et al., 1992). Interestingly, the domain two (catalytic dinucleotide-binding domain) contacts have been implicated in subunit-subunit communication important in the complex dinucleotide allosteric regulation of the enzyme.

Bascomb and Schmidt (1987) showed that the affinity of the α-homohexamer for ammonium was allosterically regulated by NADPH. The mechanism of the NADPH allosteric regulation may involve the unique α-helical domain of the α-subunit. The additional amino-terminal extension of the α-subunit may allow for contacts with the dinucleotide-binding domain two or neighboring subunits. Upon binding of NADPH at the putative amino-terminal dinucleotide-binding site, a conformational change may occur that influences the binding of ammonium to the subunit (Fig. 30). Substrate mediated conformational rearrangement of the *C. symbiosum* hexameric NAD-GDH has been shown to be an important step in the catalytic cycle of the enzyme (Stillman et al., 1992).

In addition to differences in allosteric properties, the α-subunit been shown by pulse-chase studies to be degraded three times faster than the β-subunit *in vivo* (Bascomb et al., 1987). Since the only observed differences in the two subunits is the additional 11 amino acid residues in the α-subunit, it is likely this region plays a critical role in subunit turnover. Analysis of the amino-terminal residues and structures yielded no consensus motifs that implicated

any specific degradation pathway. Previous researchers (Cock and Schmidt, unpublished data) have shown that *C. sorokiniana* NADP-GDH cDNAs subunits expressed from cDNAs in a GDH^- strain of *E. coli* are assembled into functional NADP-GDH hexamers. Therefore, it is possible to synthesize preferentially the α- or β-homohexamers in GDH^- *E. coli* for crystallagraphic analysis and mutational analyses to determine the significance of the additional protein domains of the two subunits.

The cellular abundances of the NADP-GDH mRNAs were measured throughout a 240 min induction in 29 mM ammonium medium. The induction patterns of the two NADP-GDH mRNAs were identical with the exception of a decrease observed at 240 min in the -42 nt mRNA that contrasted with an increase in the +42 nt mRNA (Fig. 27). It was evident that between 60 and 100 min a net loss of both mRNAs occurred indicating both mRNA synthesis and degradation regulate the cellular levels of both NADP-GDH mRNAs. Although both mRNAs decreased during this period, the $NADP^+$-GDH activity continued to accumulate in a linear fashion (Fig. 23). A similiar phenomena has been reported for the inducible NR mRNAs of barley (Melzer et al., 1989) and *Chlorella vulgaris* (Cannon et al., 1992). In both organisms, the NR activity continued to accumulate during induction with nitrate, whereas the NR mRNA showed an initial increase followed by a rapid decrease during the early stages of induction. Cannon et al. (1992) showed that the NR mRNA isolated from cells in early induction time periods was

resistant to cellular RNases *in vitro*, whereas NR mRNA from fully induced cells was susceptible. The results indicate that the change in NR mRNA turnover was in part due a change in mRNA stability. The degree of NR mRNA stability was inherent to the NR mRNA or factors associated with the early induced NR mRNAs. A similar mechanism might be involved in the NADP-GDH mRNA regulation and the stabilizing factor(s) may be inherent to inducible mRNAs or mRNAs of nitrogen metabolism genes.

NADP-GDH mRNA was not detected in the T_0 total RNA sample; however, NADP-GDH antigen and activity were detected indicating that protein turnover plays a significant role in the regulation of the NADP-GDH activity. Bascomb et al. (1987) showed by pulse-chase analysis that both the α- and β-subunits were turned over rapidily in *Chlorella* cells induced in 29 mM ammonium medium. The fact that no mRNA was detected in the nitrate cultured T_0 sample, but detectable levels of NADP-GDH activity and antigen were present, might reflect a difference in subunit turnover half-life that is influenced by the metabolic state of the cell.

Comparison of the induction patterns of the NADP-GDH mRNAs to the patterns of the NADP-GDH antigens did not reveal a strong correlation between a specific mRNA and antigen (Figs. 22, 27). However, the -42 nt mRNA showed a continued decrease at 240 min that correlated with a continued loss of the β-subunit antigen, whereas the +42 nt mRNA showed a marked increase that correlated with the continuous

accumulation of the α-subunit (Figs. 22, 27). By pulse-chase studies, previous researchers determined the α-subunit was synthesized five times faster than the β-subunit under comparable induction conditions (Bascomb et al. 1987). Based on the faster rate of synthesis of the α-subunit, its mRNA would be predicted to be more abundant than the β-subunit mRNA. RT-PCR analysis of the NADP-GDH mRNAs revealed the +42 nt mRNA was on average three times more abundant than the -42 nt mRNA. Although not conclusive, the aforementioned results implicate the +42 nt and -42 nt mRNAs as encoding the α- and β-subunit precursor proteins, respectively.

Comparison of the NADPH:$NADP^+$-GDH activity ratios to the subunit ratios throughout the 240 min induction in 29 mM ammonium medium revealed a correlation between a particular subunit type and activity ratio (Table 6). The peak aminating ratio occurred at 60 min when the β-subunit was the prominent antigen (but not exclusive), whereas the α-subunit was prominent when the aminating ratio was lowest. Interestingly, the aminating activity was highest when both subunits were present suggesting that the heterohexamers, formed by various combinations of the α- and β-subunits, might have a stronger influence on the catalytic activity than either subunit alone. However, the *in vivo* function of the NADP-GDH isoenzymes may not be reflected in the aforementioned *in vitro* assay, since both NADPH-GDH and $NADP^+$-GDH activities were determined in the presence of saturating amounts of substrates. This substrate saturation

might particularly affect the α-homohexamer that shows a decreased affinity for ammonium as the NADPH concentration is increased (Bascomb and Schmidt, 1987).

The induction patterns of the NADP-GDH α- and β-subunits observed in this research (Fig. 21) differed from the patterns reported by previous researchers (Prunkard et al., 1986; Bascomb and Schmidt, 1987). Previous studies showed that *C. sorokiniana* cells induced in 29 mM ammonium medium accumulated both the α- and β-subunits for the first 120 min with only the β-subunit accumulating thereafter (Bascomb and Schmidt, 1987). In this study, both subunits accumulated for the first 40 min after which the β-subunit continued to decrease, whereas the α-subunit increased rapidly for the remainder of the induction period (Fig. 22). Furthermore, cells cultured continuously in 29 mM ammonium medium synthesized primarily the α-subunit (Fig. 14), whereas previous researchers reported the β-subunit primarily accumulated at this ammonium concentration (Yeung et al., 1981; Bascomb and Schmidt, 1987). *Chlorella* cells cultured in 1 mM ammonium medium accumulated only the α-subunit (data not shown) which is consistent with the results of Bascomb and Schmidt (1987).

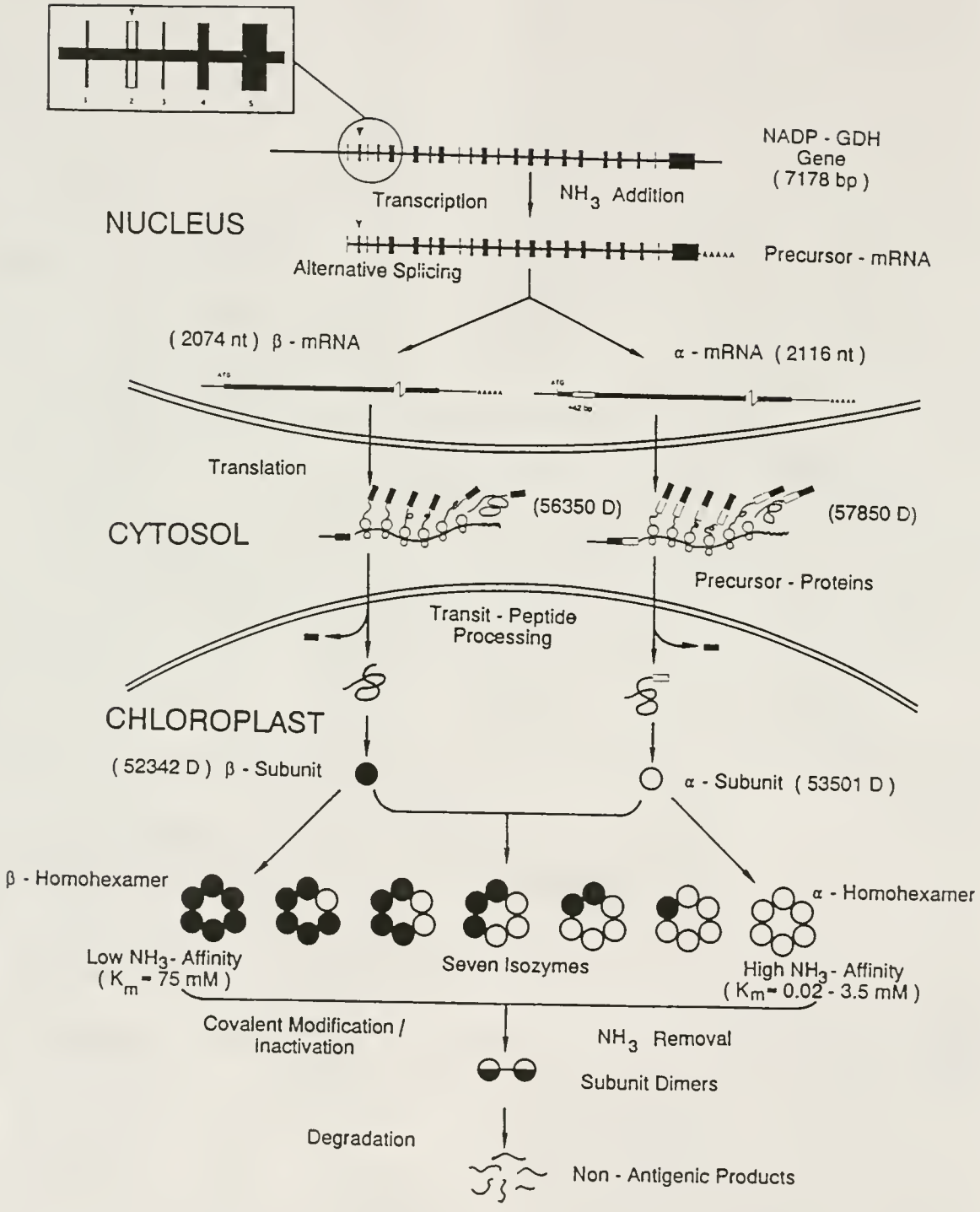
These apparent inconsistencies imply the regulation of the NADP-GDH isoenzyme pattern is likely influenced by multiple metabolic signals rather than strictly the nitrogen status of the cells. Prunkard et al. (1986) showed that the NADP-GDH α:β-subunit ratio was influenced by both the carbon

source and light conditions under which the *C. sorokiniana* cells are cultured. GDH activity and isoenzyme pattern in *Nicotiana* (Maestri et al., 1991) and carrot (Athwal, 1994) cell suspension cultures are influenced by nitrogen and carbon source as well as energy status of the cell. It is hypothesized that the suspension cultures may exhibit a regulatory mechanism similiar to the catabolic repression observed in prokaryotes and eukaryotes (Magasanik, 1961; Polakis and Bartley, 1965) and only recently detected in higher plants. In addition, the GDH levels and isoenzyme patterns have been shown to be influenced by growth regulators and environmental stress conditions in higher plants (Srivastava and Singh, 1987). Since the *C. sorokiniana* NADP-GDH isoenzymes are regulated by both carbohydrate and nitrogen metabolism, it is possible that the regulatory signal involves the common metabolic link between the two pathways, glutamate. With the molecular probes provided in this research, it should be possible to further elucidate the metabolic regulation of the NADP-GDH isoenzymes.

The model presented in Figure 31 was proposed to summarize the data presented herein. The *C. sorokiniana* nuclear genome possesses a single NADP-GDH gene in which the transription rate is increased upon the addition of ammonium to the culture medium. The intracellular inducer is not likely ammonium, but rather an organic metabolite (eg. glutamate) derived from a nitrogenous or non-nitrogenous

compound whose intracellular pool is influenced by both carbon and nitrogen metabolism. The resulting pre-mRNA undergoes alternative processing that is either directly or indirectly regulated by the organic metabolite. Under conditions that favor the α-homohexamer, the pre-mRNA is processed to 22 exons that yields the +42 nt NADP-GDH mRNA (2116 nt) that encodes a 57850 D precursor protein. Under conditions favoring the β-homohexamer, the pre-mRNA is processed to 23 exons that yields the -42 nt NADP-GDH mRNA (2074 nt) that encodes a 56350 D precusor protein. Upon import into the chloroplast, the 57850 and 56350 D precursor proteins are processed by a general SPP to the mature α-subunit (53501 D) or β-subunit (52342 D), respectively. The mature subunits are assembled in the chloroplast stroma to homo- and heterohexamers with different substrate affinities and allosteric properties. Upon removal of the inducer from the culture medium, the subunits comprising the isoenzymes are rapidly inactivated by covalent modification to form subunit dimers that target them for degradation by chloroplastic proteases (Bascomb et al., 1987). Although the proposed molecular regulation has not been demonstrated unequivocably, the proposed model provides a scaffold to direct future research into the regulation of the *C. sorokiniana* NADP-GDH isoenzymes.

Figure 31. Model for the regulation of the *C. sorokiniana* chloroplastic NADP-specific GDH isoenzymes. In the diagram of the model, black boxes represent exons, the white area, denoted by the arrow over exon two, indicates the alternatively processed 42 nt auxon. The mRNA sizes do not include the poly(A) tail. The white box in the 57850 D precursor protein represents the additional 11 amino-terminal residues of the α-subunit. The large open and filled circles depict the mature α- and β- subunits, respectively.


NADP - GDH
Gene
(7178 bp)
Transcription
NH3 Addition
NUCLEUS
Precursor - mRNA
Alternative Splicing
(2074 nt) β - mRNA
α - mRNA (2116 nt)
Translation
CYTOSOL
(56350 D)
(57850 D)
Precursor - Proteins
Transit - Peptide
Processing
CHLOROPLAST
(52342 D) β - Subunit
α - Subunit (53501 D)
β - Homohexamer
α - Homohexamer
Low NH3 - Affinity
(Km = 75 mM)
Seven Isozymes
High NH3 - Affinity
(Km = 0.02 - 3.5 mM)
Covalent Modification /
Inactivation
NH3 Removal
Subunit Dimers
Degradation
Non - Antigenic Products

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Philip W. Miller was born in Cincinnati, OH, on August 9, 1962. He graduated from Lawrenceville Township High School in Lawrenceville, IL, in May 1980, and entered Bob Jones University in August of that year. He received a Bachelor of Science degree in biology, with a minor in English, in May 1984. After graduation, he worked as a genetic research assistant for Agrigold Seed Company and as a regional sales manager for PepsiCo Corporation before entering the Master of Science program at Appalachian State University in August of 1987. In January of 1990, he transferred from Appalachian State University and entered the Department of Microbiology and Cell Science at the University of Florida where he began studies towards the Doctor of Philosophy degree.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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